PYOCINES AND THE TREATMENT OF PSEUDOMONAS AERUGINOSA INFECTIONS

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SUMMARY

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1. The aim of this investigation was to study the interaction of different kinds of pyocine with a single sensitive indicator strain of Pseudomonas aeruginosa, and to examine the possible therapeutic applications of these pyocines to infections caused by the indicator strain.

2. After surveying the literature concerning Ps. aeruginosa, with particular emphasis on the pathogenicity of the organism, the past and present therapy of Ps. aeruginosa infections was discussed.

3. The literature on the pyocines was reviewed and it was clear that two main groups of pyocines were distinguishable; the high and low molecular weight groups, and within the high molecular weight pyocines, two structural types were defined; contractile and filamentous. Members of the low molecular weight group were referred to as small pyocines.

4. Ninety-four strains of Ps. aeruginosa were examined for pyocine production against two selected indicator strains, Ps. aeruginosa 6A and P14. Thirteen strains which produced pyocines with properties characteristic of each of the three types of pyocine were selected and examined in greater detail.

5. The inducibility of the strains was examined and the effects of heat, trypsin and ultracentrifugation on the extracted pyocines were investigated. Electronmicroscopy was used to confirm the classification of the pyocines.

6. Three pyocinogenic strains were selected for further investigation; one (5893) which produced contractile pyocines, another (5882) which produced filamentous pyocines, and a third (H108) which produced small pyocines. These pyocinogenic strains were defined with respect to their pyocine activity against indicator strain P14 only and this indicator strain alone was used throughout the remainder of the study. The pyocines were numbered according to their producer strain, e.g. pyocine 5893 was the pyocine produced by strain 5893 and inhibitory to indicator strain P14.

7. Various methods for purifying and concentrating the pyocine preparations were examined and precipitation of the proteins with ammonium sulphate proved the most satisfactory.

8. Mouse toxicity testing of pyocine preparations at various degrees of purity revealed that in some forms, certain pyocine preparations caused adverse effects. Uninduced, low activity preparations of the pyocine were more lethal, suggesting that factors other than pyocine were responsible for toxicity, but examination of fractions of an induced preparation <u>in vivo</u> favoured the pyocine itself as the toxic agent.

9. In view of the toxicity problems, another pyocinogenic strain (1577), which produced contractile pyocines inhibitory to indicator strain P14, replaced the toxic pyocine (5893).

10. An extended pyocine purification scheme was devised that included ion-exchange chromatography. The method gave satisfactory results with the high molecular weight pyocines but the small pyocine proved more problematic.

11. The interactions between the pyocines and cultures of strain P14 were examined <u>in vitro</u> and the effects of dosage and incubation time on the survival of P14 cells were studied.

12. The pyocines were examined in mice, alone, and in the presence of infections caused by strain P14. When the pyocines were injected into mice by various routes, inhibitory activity against strain P14 could be identified in the serum for several hours.

13. High molecular weight pyocines given intraperitoneally in the presence of a lethal dose of strain P14 administered by the same route, were unable to prevent the fatal outcome of the infection unless they were given before or simultaneously with the bacteria. The small pyocine preparation had no protective effect.

14. Infection of experimentally-induced burns with strain P14 was examined; although colonisation of the burn was easily established, lethal infections were more difficult to achieve. The effects of topical application of the high molecular weight pyocines on infected burns were

examined and it was concluded that the pyocines did not improve the chances of survival of burned, infected mice.

15. Despite the problems of pyocine resistance of strain P14 recognised *in vitro*, this did not appear to be the cause of failure of pyocine therapy *in vivo*.

16. A single, intraperitoneal dose of pyocine was sufficient to cause the production of pyocine-neutralising antibody, but topically-applied pyocine did not elicit an antibody response.

17. The future of pyocine therapy was discussed.

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INTRODUCTION

CHAPTER I

PSEUDOMONAS AERUGINOSA

A bacterium responsible for the bluish discolouration of pus was isolated by Gessard in 1882 and named by him Bacillus pyocyaneus. In 1889, Charrin (cited by Forkner, 1960) clearly demonstrated the pathogenicity of this organism in rabbits and around the turn of the century there were several reports of the isolation of Bacillus pyocyaneus from various human infections. There has been a lengthy controversy over the correct nomenclature for this organism, which has been variously called Bacillus pyocyaneus, Pseudomonas pyocyanea and Pseudomonas aeruginosa. Hugh and Lessel (1967) discussed the various forms and concluded that the name Pseudomonas aeruginosa was the most acceptable. Despite its recognised pathogenicity, Ps. aeruginosa was not prominent as a cause of human infections in the first part of this century when Gram-positive cocci and tubercle bacilli were providing more serious menaces to human life (Finland, Jones and Barnes, 1959). Although Groves (1909) said of Ps. aeruginosa "now this is a germ we are too apt to treat lightly", it was some forty years later following the introduction of antibiotics, particularly the broad-spectrum antibiotics, that a marked increase in infections caused by the Gram-negative bacilli was seen (Rogers, 1959; Asay and Koch, 1960; Barber, 1961). Ps. aeruginosa has played a leading role in this increase, probably because it is a ubiquitous saprophyte which is naturally resistant to many antibacterial agents and it has a great capacity for survival in

environments such as disinfectants, ointments and humidifiers. It is an excellent example of an opportunist pathogen that attacks patients who are already debilitated and whose natural defences are deficient.

General characteristics of Pseudomonas aeruginosa

Ps. aeruginosa is a Gram-negative bacillus belonging to the family Pseudomonadaceae. The genus Pseudomonas consists of many species which are predominantly free-living in soil or water, but several are plant pathogens and a few including Ps. aeruginosa, are pathogenic to animals. Ps. aeruginosa is the type species of the genus and is an aerobic or facultatively anaerobic organism whose optimum growth temperature is 37°C. The cells measure about 0.5 by 1.5μ and are motile by means of one to three polar flagella (Bergey's Manual, 1957).

Ps. aeruginosa can be isolated in several distinct colonial forms (Phillips, 1969) and a single culture may alter its colonial type; a phenomenon known as dissociation (Gaby, 1946; Zierdt and Schmidt, 1964). Lysogeny is common in Ps. aeruginosa and may also result in phenotypic colony changes. Some workers have suggested that the different colonial forms differ in their sensitivity to various agents such as antibiotics and bacteriophage (Zierdt and Schmidt, 1964; Homma, 1971). Although Ps. aeruginosa is frequently recognisable by its colonial appearance, other identifying features include the production of a greenish-blue pigment, pyocyanin; the ability of the organism to grow at 42°C, to produce cytochrome

oxidase, and to produce slime in a defined liquid medium containing 2-ketogluconate. The analysis of the slime has produced contradictory results but it appears that the main constituent is a polysaccharide composed chiefly of glucose. Deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and hyaluronic acid are also present together with small amounts of protein, rhamnose and glucosamine (Brown, Scott, Foster and Clamp, 1969). The slime layer is antigenic and has toxic properties that will be discussed later.

The mucoid form of Ps. aeruginosa is one of the less common colonial types but shows a particular association with pulmonary infections in children with cystic fibrosis, (Zierdt and Schmidt, 1964; Doggett et al., 1965). Some reports propose that the mucoid cells are encapsulated while others suggest that the mucous material is less firmly bound to the cell. The mucoid strains of Ps. aeruginosa do not simply produce larger amounts of the characteristic slime described above, but elaborate a completely different substance composed of uronic acids and resembling alginic acid (Carlson and Matthews, 1966).

The cell walls of Ps. aeruginosa resemble those of other Gram-negative bacteria. Adjacent to the cytoplasmic membrane is a layer of murein surrounded by a complex membranous structure of lipoprotein and lipopolysaccharide (LPS). The lipids of the cell wall consist of two types; those that are firmly attached to the protein or polysaccharide and those that are loosely bound. The latter are composed mostly of free fatty acids and phospholipids and are associated with magnesium and calcium ions (Bobo and Eagon, 1968). These divalent cations are also thought to play a

role in stabilising the surface structure of Ps. aeruginosa both by linking polysaccharide subunits and by stiffening the lipoprotein component (Zimelis and Jackson, 1973). The LPS constitutes the somatic antigen and provides receptor sites for some bacteriophages and pyocines.

Ps. aeruginosa is well equipped for its role as an opportunist pathogen. It is highly versatile and can adapt to a variety of situations. Being primarily a saprophyte, the organism has simple nutritional requirements and a broad spectrum of enzymes enabling it to obtain carbon and energy by the oxidation of a wide variety of organic compounds; for example, from the hydrocarbons of jet fuel (Bushnell and Haas, 1941) and from poor quality distilled water (Favero et al., 1971). Benzalkonium chloride is a quaternary ammonium compound commonly used as a preservative in pharmaceuticals and Ps. aeruginosa is capable of growing in such preparations, where it is thought to utilise the benzalkonium chloride molecule as a carbon and energy source and destroy its antimicrobial activity (Adair, Geftic and Gelzer, 1969). Such contaminated preparations provide dangerous sources of infection. Ps. aeruginosa can survive in a range of environments although it has a preference for the moist over the dry and it rapidly colonises environments that are contaminated with small amounts of organic materials. It is the adaptability of Ps. aeruginosa that is the keynote to its success as an opportunist pathogen.

Pathogenicity of Pseudomonas aeruginosa

Ps. aeruginosa elaborates a large variety of extracellular products some of which, the pyocines, are antagonistic to members

of the same genus, some have antibiotic activity against other genera, and some have been implicated in the pathogenicity of the organism. The last group includes the pigments, slime, endotoxin, extracellular enzymes and exotoxins, and the parts they may play in the pathogenicity of Ps. aeruginosa are reviewed here.

Pigments

The blue-green pigment of Ps. aeruginosa was observed before the organism was even isolated (Fordos, 1860). In 1953, Cruickshank and Lowbury studied the effect of pyocyanin on tissue cultures of human skin cells and leucocytes and found it to be toxic to the former at levels greater than 0.0024 mg/ml and to the latter at 0.08 mg/ml. These authors calculated the amount of pyocyanin that might be present in the infected burn exudate and suggested that the pigment would be capable of causing toxic effects in vivo. Leucocytes were less sensitive and are probably not affected in vivo. Thus it was postulated that pyocyanin might have a role in the local toxicity in surface infections.

More recent work (Armstrong, Stewart-Tull and Roberts, 1971) has shown that it is a breakdown product of pyocyanin, 1-hydroxy-phenazine, that is the active principle and this compound inhibits mouse liver mitochondrial respiration in vitro. These workers postulate that in a surface infection with Ps. aeruginosa, 1-hydroxyphenazine would be produced and impair or kill the macrophages, thereby removing one of the body's front-line defences. This might be followed by destruction of the tissue cells and the permeation into the bloodstream of the 1-hydroxyphenazine which would reach the liver cells and inhibit their mitochondria, eventually causing cytolysis, and death of the animal. However

Armstrong and her colleagues accept that this is probably not the only factor involved in pathogenicity.

Slime

The slime material commonly produced by strains of Ps. aeruginosa in fluid cultures has been implicated in pathogenicity (Liu, Abe and Bates, 1961); it contains highly polymerised DNA and Callahan, Beyerlein and Mull (1964) showed that as the DNA was depolymerised, the material became increasingly toxic to mice. These workers suggested that there were enzymes present in the mouse that were capable of hydrolysing polymerised DNA. Thus the slime may play a toxic role in the pathogenicity of Ps. aeruginosa. It has been suggested that the hyaluronic acid component of the slime, which adheres firmly to the cell surface, may function as a microcapsule in a manner analogous to the hyaluronic acid capsules of Gram-positive organisms (Brown et al., 1969).

Mucoid strains of Pseudomonas aeruginosa

The mucus material produced by mucoid strains of Ps. aeruginosa has quite a different chemical composition from the more common slime and it has been reported as being toxic to chick embryos (Cundy and Mattson, 1969) and to mice (Doggett and Harrison, 1969), when administered in a purified form. The material also possesses antiphagocytic activity (Schwarzmann and Boring, 1971).

Endotoxin

Early workers expected that the endotoxin of Ps. aeruginosa would cause similar effects on the host to those caused by the endotoxins of the Enterobacteriaceae. However Michaels and

Eagon (1969) found significant differences between the LPS isolated from Ps. aeruginosa and from the Salmonella-Escherichia group. The backbone of the LPS appears to be the same in the two groups but the side-chains of the Ps. aeruginosa LPS are much less complex and involve a smaller variety of carbohydrates. Liu et al. (1961) showed that cells of Ps. aeruginosa were relatively non-toxic when injected into mice and Hall et al. (1968) agreed that the cells did not stimulate an endotoxic reaction. However if the protein portion of the endotoxin is extracted and tested in vivo it is capable of eliciting a Shwartzman reaction and a pyrogenic response (Johnston, Morris and Berk, 1967; Homma, 1971). But Gorrill (1965) and later Klynn and Gorrill (1967) suggested that endotoxaemia was the cause of death in mice that were injected with large doses of Ps. aeruginosa intravenously. Cundy and Mattson (1969) reported the toxicity to mice of endotoxin extracted from a mucoid strain of Ps. aeruginosa.

Extracellular enzymes

Ps. aeruginosa produces a battery of extracellular enzymes including protease, gelatinase, elastase, lecithinase, lipase, acid and alkaline phosphatase and coagulase.

In 1953, Yow and Townsend reported the problems of applying skin grafts to burns infected with Ps. aeruginosa. They isolated strains of the organism that had potent proteolytic activity and could lyse blood clots, plasma clots, coagulated serum and coagulated human skin and muscle. In experiments with rabbits, Fisher and Allen (1958a) showed that intracorneal inoculation of a

virulent culture of Ps. aeruginosa caused corneal ulceration. But the same effect could be demonstrated by inoculating a cell-free culture filtrate from the virulent strain. These workers correlated the corneal damage with protease elaborated by the organism, particularly collagenase activity (Fisher and Allen, 1958b). They suggested that the enzyme was adaptive and was induced by the presence of suitable protein substrates in the eye.

Liu et al. (1961) fractionated cultures of Ps. aeruginosa and tested each fraction in mice for its ability to produce pathological effects. They found that the extracellular enzyme fraction, particularly protease and lecithinase played an important role in surface lesions and also contributed to the lethality of a generalised infection. But later Liu and Mercer (1963) found that a strain must be able to grow in the serum of the test animal as well as to produce extracellular enzymes in order to be virulent to that animal.

Carney and Jones (1968) compared the production of extracellular enzymes by strains of Ps. aeruginosa that caused septicaemia and death in burned mice (virulent strains) with the production by strains that did not cause septicaemia and death (avirulent). In vitro, the virulent strains produced more of each enzyme tested than did the avirulent strains. The maximum enzyme production was around the fifth day of culture and this is comparable with the time at which experimentally burned and infected mice die of septicaemia.

Klynn and Gorrill (1967) and later Muszynski (1973) defined the virulence of a strain as its ability to kill mice after intraperitoneal administration. They found that strains of Ps. aeruginosa could be classified into high, medium and low virulence

groups. The high virulence strains exhibited the greatest overall extracellular enzyme activity (when tested *in vitro*) and the best correlation was between protease production and virulence.

Exotoxins

It has been difficult to show that extracellular enzymes are actually produced *in vivo* and thus their importance in the pathogenicity of *Ps. aeruginosa* infections has not been confirmed. Liu (1966) found that when a virulent strain of *Ps. aeruginosa* was grown in rabbit serum, lecithinase was not produced and the protease production was very low. He isolated a "lethal toxin" from the excised skin of a rabbit that had been injected intracutaneously with a strain of *Ps. aeruginosa*, and showed it was lethal to mice when administered intraperitoneally. The toxin could not be identified with any in-vitro enzymatic activity that had been described. A similar toxin could be produced *in vitro* by suitable manipulations of the growth conditions. This toxin appears to be a protein with a molecular weight in the range of serum globulin and without enzymatic activity. In-vivo experiments showed that the toxic factor could kill mice within a few hours but no significant histopathological changes were seen at necropsy.

In later studies, Liu (1973) found that a strain could produce several types of exotoxins, one of which, exotoxin A, was relatively stable and therefore more easily purified. When tested in mice, a purified preparation of exotoxin A contained 8,000 LD_{50} per mg protein, which is of the same order as the exotoxins of other bacteria (Liu, Yoshii and Hsieh, 1973). Antitoxic sera produced against exotoxin A could prevent the lethal effects of infection

caused by strains of Ps. aeruginosa whose somatic antigens were unrelated to the toxin-producing strain, provided the exotoxin of the lethal strain was serologically identical to exotoxin A (Liu and Hsieh, 1973). The latter finding suggests that the LPS somatic antigen is not an important factor in the pathogenesis of Ps. aeruginosa.

Injection of the exotoxin into dogs (Atik et al., 1968) resulted in two types of reaction; an immediate anaphylactoid reaction, and a late hypotensive reaction. The dogs usually recovered from the first, only to die of the second. In some ways these reactions resemble those following injection of endotoxin but differ in that invasion of the bloodstream by the organisms was not necessary. When the toxin is added to African green monkey-kidney cell cultures, it binds immediately to the cells but there is then a lag of about five hours before irreversible damage occurs (Pavlovskis and Gordon, 1972). The toxin was found to damage the electron-transport chain in mitochondria and whole cells from mouse liver, and induced mitochondrial swelling, suggesting changes in membrane permeability (Pavlovskis, 1972). Damage to mouse liver mitochondria was also induced by the pigment breakdown product 1-hydroxyphenazine as already noted.

In summary, of the factors that have been implicated in the pathogenicity of Ps. aeruginosa, endotoxin seems to play a very small role. The pigments may contribute to the deleterious effects but the extracellular enzymes and the exotoxins seem to be the main contenders. The slime may also be toxic and may play a role in protecting the organisms from the hosts' defences.

Experimental Pseudomonas aeruginosa infections in animals

One of the problems encountered in the study of the pathogenicity of an organism is that the virulence factors may be produced only *in vivo*. In recent years evidence has accumulated that many bacterial species grown in infected animals are chemically and biologically different from their counterparts grown *in vitro* (Smith, 1972). The exotoxin and extracellular enzymes of *Ps. aeruginosa* described by various workers (Liu *et al.*, 1961; Liu, 1966; Carney and Jones, 1968; Pavlovskis, 1972) may play a role in damaging the host but the mere presence of large bacterial populations may also harm the host by producing emboli or depleting the host tissue of its essential nutrients.

Bartell, Orr and Garcia (1968) described an in-vivo experimental system which allowed quantitation of the bacterial population during the course of an infection. Mice were inoculated intraperitoneally with a dose of *Ps. aeruginosa* (2.5×10^8 organisms) which resulted in 80-100% mortality within 48 hours. Smaller doses of organisms had no adverse effect on the animals. The mice were killed at intervals after inoculation and the numbers of organisms in peritoneal washings, blood, lung, liver, kidney and spleen were determined. Bartell *et al.* (1968) suggested that their results showed three distinct events during the course of the infection. Firstly the lethal factors were expressed. Secondly there was a significant increase in the numbers of bacteria present and lastly the mice died. The rapid course of the fatal infection (death usually occurred within 8-12 hours of inoculation) suggested a toxic death, but preformed toxin could not be demonstrated in the

inoculum and it was implied that the toxin was elaborated in the animal after the injection of the bacteria. Bartell et al. (1968) used specific bacteriophage to control the numbers of bacteria at various times during the course of infection. They found that unless the phage were administered within 30 min. of inoculation of the bacteria, the mortality of the mice was unaffected. Thus the authors suggested that the increase in bacterial numbers did not contribute to the lethality of the infection but that some toxic substance was implicated. These results support the finding (Liu, 1966; Pavlovskis, 1972; Liu, 1973) that exotoxins are the chief virulence factors of Ps. aeruginosa. However the experimental model used by Bartell et al. (1968) where a massive dose of organisms is introduced directly into the peritoneal cavity, is not typical of naturally occurring Ps. aeruginosa infections in humans. Smaller numbers of Ps. aeruginosa injected intraperitoneally can produce lethal infections in mice if the animals are pretreated with cortisone (Rosenthal, Millican and Rust, 1957) or 6-mercaptopurine (Whittaker, 1971), or if the infected dose is suspended in mucin (Olitzki, 1948; Stephens, 1959).

In an effort to examine an experimental infection that more closely resembled a human infection, Klynn and Gorrill (1967) studied the development of Ps. aeruginosa pyelonephritis in mice. They attempted to establish infection by intravenous inoculation of the organisms but found that in healthy mice the abscess-forming dose was very close to the lethal dose. However where death was avoided, the mice did develop typical pyelonephritis which imitated

the human infection, but conclusions on the possible virulence factors of Ps. aeruginosa involved were not reached.

The animal studies described above are based on intraperitoneal or intravenous administration of large doses of Ps. aeruginosa frequently resulting in rapid death of the animal. But such appreaches are not satisfactory for the study of the pathogenic mechanisms involved in surface infections such as those of burns. Ps. aeruginosa infections of experimentally-produced burns in animals have been studied by several workers. Mice and rats are usually used and the burns are produced either by immersing part of the anaesthetised animal in boiling water or steam (McRipley and Garrison, 1964; Teplitz et al., 1964; Skornik and Dressler, 1970) or by applying a heated brass block to the back of the animal (Jones, Jackson and Lowbury, 1966). The size of the burn is so designed that the animals survive unless they are deliberately infected with Ps. aeruginosa.

Teplitz and his colleagues (1964) recognised the importance of pseudomonas septicaemia as a common cause of death following infection of burns and they devised an experimental model to try and elucidate the pathogenesis of pseudomonas burn wound sepsis. Rats were burned over 20% of their body surface area by scalding the skin in boiling water for 10 s. This resulted in a full-skin thickness burn whose natural course was characterised by sloughing of the eschar and formation of scar tissue. The burns were seeded with 1 ml of a culture of Ps. aeruginosa containing 4×10^8 bacteria and the rats were killed at intervals after infection. The course of the bacteria during the infection was followed by estimating the number of organisms in the excised burned tissue and in the blood

and organs. Up until 24 hours after infection, the Ps. aeruginosa remained on the surface of the eschar but after this time, intra-eschar counts began to rise and by the fourth day the bacteria had invaded damaged muscle tissue beneath the burn. By the eighth day the bacteria had invaded the viable tissue surrounding the burn and splenic and blood cultures were also positive for Ps. aeruginosa. None of the rats developed visceral lesions before the seventh day, but thereafter these increased in frequency and were found most commonly in the lungs. Teplitz et al. (1964) suggested that their findings bore a close resemblance to the human infection. The rats frequently became ill before positive blood cultures were observed and the authors suggested that this was due to the production and liberation of toxic factors by the Ps. aeruginosa in the burn wound before septicaemia developed.

Stone (1966) fractionated a culture of Ps. aeruginosa into two extracts; the slime layer and the somatic layer. When these fractions were administered to mice by the intraperitoneal route, the somatic layer was not lethal, but the slime layer contained three groups of lethal toxins. One was a haemolysin that produced marked haemolytic anaemia when injected into rats. The second group of exotoxins consisted of cytotoxins which produced areas of necrosis when injected subcutaneously into rats, and the third group of toxins inhibited certain functions of the host's reticulo-endothelial system. The latter group may be those of the type described in in-vitro studies by Liu (1973). Stone (1966) postulated that the inhibition of host defence mechanisms by the Ps. aeruginosa toxins might be sufficient to precipitate toxaemic death of burned and infected patients or at least to facilitate

invasion of the organisms into the bloodstream. This could help to explain the observation of negative blood cultures in the presence of overt burn wound sepsis.

Skornik and Dressler (1970, 1971) used burned rats to examine the development of respiratory tract complications following burn injury. After infection of the burns with Ps. aeruginosa they found an apparent depression in the reticulo-endothelial response, particularly in the lung macrophage mechanism, thus supporting Stone's observations (1966) on the inhibition of the host defences. The inability to clear bacteria from the lungs correlated with the numbers of organisms in the sub-eschar tissue. Death from infection did not occur unless the bacterial count in the fascia and muscle underlying the eschar rose to about 10^5 organisms per g of tissue.

In a series of experiments with mice, Jones and his colleagues evolved an experimental model for pseudomonas burn infection. The animals were burned by the application of a standard brass block previously heated in boiling water (Jones et al., 1966). Following infection of the burns with a virulent strain of Ps. aeruginosa, the mortality rate was 71% and the mean time to death of these mice was 6.2 days. Carney, Dyster and Jones (1973) compared the activities of two strains of Ps. aeruginosa on mice burned by this method; one strain was lethal to burned mice, but the other was not. During the first 48 hours after the burns had been seeded with the lethal strain, considerable bacterial multiplication occurred on the surface of the skin and in the hair follicles. The bacteria then began

to invade and multiply deeper in the dermis and in the dorsal muscles. Death due to septicaemia occurred around the fifth day after infection. In contrast, the non-lethal strain remained localised in the outer regions of the burned skin and did not invade the deeper tissues. The invasive properties of these two strains of Ps. aeruginosa were thought to be associated with their ability to produce extracellular enzymes, particularly proteases (Carney et al., 1973). Carney and Jones (1968) had previously shown that protease production occurs at a maximum from the fifth day of culture in vitro and it may be significant that invasion of the deeper tissues of the burned animal, culminating in death, also occurred around the fifth day.

The burn infection model, in its various forms, has proved very useful in the study of pseudomonas infections. At first sight it may appear somewhat artificial but in fact it mimics the human burn infection very closely. The results suggest that the pathogenicity of Ps. aeruginosa in burns is due partly to the toxigenic and partly to the invasive properties of the organisms. In vivo, Ps. aeruginosa appears to produce a number of exotoxins in conjunction with the slime and one or more of these impairs the host defence mechanisms. The extracellular enzymes that were described in vitro (Liu et al., 1961; Carney and Jones, 1968) play a role in the invasion of the bacteria from the surface into deeper tissues.

Infections caused by Pseudomonas aeruginosa

Ps. aeruginosa is primarily a saprophyte but its pathogenic properties have already been stressed. The hospital provides a delightful environment for an opportunist pathogen such as Ps. aeruginosa; debilitated patients abound, moist niches for the organism to skulk in, and an abundance of antibiotics and disinfectants which serve to reduce the numbers of competing organisms while the resistant Pseudomonas survives triumphant.

Outside the hospital Ps. aeruginosa rarely troubles healthy humans and carriage rates of the organism in the population are low (Lowbury and Fox, 1954) and colonisation of the gastro-intestinal tract is difficult to achieve experimentally in humans (Buck and Cooke, 1969). But a high proportion of hospital patients carry Ps. aeruginosa in the bowel (Stoodley and Thom, 1970) and contaminated food and medicaments have been considered as possible reservoirs from which a patient may become colonised (Shooter et al., 1969). However, most Ps. aeruginosa infections in hospitals are thought not to be endogenous in origin.

In his monograph on pseudomonas infections (1960), Forkner wrote "Given the proper predisposing circumstances, there is scarcely a region of the body exempt from infection with Pseudomonas aeruginosa." The "predisposing circumstances" are almost always constituted by a primary debility in the patient and infections are uncommon in other circumstances, except when the bacteria are directly introduced e.g. by lumbar puncture or

in eye surgery. In other words, Ps. aeruginosa takes the opportunity of infecting patients whose defence mechanisms are already under par.

When considering a list of infections caused by Ps. aeruginosa it is hard to find a part of the body that escapes a mention. The primary sites of colonisation are the skin and mucous membranes and the organisms may remain localised or disperse to invade other organs.

Skin infections with Ps. aeruginosa often occur in patients with neoplastic disease or those receiving large amounts of antibiotics. Conditions such as green-nail syndrome and toe-web infection are localised infections resulting from colonisation by Ps. aeruginosa under conditions of increased moisture, and are not serious. However the skin lesions constituting ecthyma gangrenosum are viewed much more seriously and are virtually pathognomonic of pseudomonas septicaemia. The lesions evolve in a characteristic manner: Small vesicles develop, typically isolated from one another, oedema is followed by erythema and the fluid contents of the vesicle become milky or haemorrhagic and burst spontaneously leaving a necrotic area surrounded by induration (Hall et al., 1968; Dorff et al., 1971). The entire sequence may be completed within 12 hours. The bacteria proceed to invade the walls of the veins and later the arterial walls and septicaemia rapidly develops. Blood cultures may be positive for Ps. aeruginosa before the ecthyma gangrenosum is manifest but more often the skin condition is noted first. Whichever way, the fatal course of a septicaemia is rapid and

effective therapy is difficult to achieve unless the condition is diagnosed in its early stages.

Forkner et al. (1958) examined 23 cases of septicaemia of which 22 were fatal. Twenty-one of the 23 patients had malignant neoplastic disease. The median duration of life after the first positive blood culture was four days. Similarly, Cooper (1967) reported 34 deaths among 35 children with pseudomonas septicaemia. He found that the tendency to infection in the face of a primary debility was not universal but the ages of his patients may have contributed to their disadvantage.

Ps. aeruginosa endocarditis is uncommon but when it occurs it is very difficult to treat. Blood-borne spread of the organisms may lead to localisation of bacteria on the heart valves and from here complications of the central nervous system frequently develop. Ps. aeruginosa may implant on normal as well as abnormal heart valves (Carruthers and Kanokvechayant, 1973) and the infection is often fatal. A diagnosis of endocarditis is difficult to make in a patient who survives and is usually assumed on the basis of prolonged bacteraemia without any other apparent source.

Ps. aeruginosa is a menace in burn infections and frequently contributes to the fatal outcome. In 1965, Lindberg et al. reported that 60-70% of deaths in burned patients due to septicaemia were attributable to pseudomonas burn wound sepsis. The burn provides a large surface area, rich in nourishment and

susceptible to colonisation by many organisms including Ps. aeruginosa. Colonisation is a nuisance because it delays wound healing and provides a dangerous source of organisms from which other patients may be infected. But the pseudomonads may not be content with colonisation alone and often invade deeper into the burned areas and into the adjacent viable tissue and eventually enter the bloodstream. Septicaemia develops rapidly and is often fatal. Ironically, the presence of Ps. aeruginosa in a burn may have some advantage. Stone, Given and Martin (1967) observed that homografts survived longer in patients whose burns were infected with Ps. aeruginosa. This may be due to the production of toxins by the organisms which depress the host defence mechanisms (Stone, 1966).

A disturbing number of Ps. aeruginosa infections, which should be preventable, result from the use of contaminated fluids or equipment. Eye infections with Ps. aeruginosa, caused by instillation of contaminated eye drops or saline, have been reported. Ps. aeruginosa is a particularly dangerous pathogen to the eye, where it rapidly causes corneal ulceration which often leads to loss of the eye (Ayliffe et al., 1966). Contaminated fluids or equipment have also been incriminated in cases of meningitis following spinal anaesthesia (Weinstein and Perrin, 1948). Like endocarditis, infection of the meninges with Ps. aeruginosa is uncommon but important because of the high mortality rate. Meningitis may also develop as a result of haematogenous spread of the bacteria (Forkner, 1960).

Contamination of needles and syringes was thought to be the cause of two cases of osteomyelitis and vertebral disc infection in heroin addicts reported by Selby and Pillay (1972). In such circumstances, heroin is usually diluted in unsterile conditions before administration and injection is made without prior cleansing of the skin and often with a syringe and needle shared by a group of addicts, so it is hardly surprising that infections occur.

Catheterisation is almost a password for the development of urinary tract infections. Because of the abundance of Ps. aeruginosa in the hospital environment and its ability to survive in disinfectants which may be used for storing catheters, it is often the causative agent of urinary tract infections. As a result, pyelonephritis may develop and be extremely difficult to treat. Improvement in catheterisation techniques (Pyrah et al., 1955) and the use of disposable catheters has to a large extent reduced the occurrence of this form of Ps. aeruginosa infection.

Pseudomonas pulmonary infections frequently result from the use of contaminated respiratory equipment (Bassett, Thompson and Page, 1965; Phillips and Spencer, 1965; Tinne et al., 1967). Such equipment often incorporates a water reservoir which provides an ideal environment for Ps. aeruginosa to lurk in. From the contaminated reservoir, aerosols containing large numbers of viable bacteria are generated and pass directly into the patient's respiratory tract. Complex pieces of apparatus, such as

respirators, are a hallmark of modern medicine but they are notoriously difficult to sterilise and Ps. aeruginosa is adept at taking advantage of this situation. The patients at greatest risk are those with chronic lung disease or other debilitating states (Tillotson and Lerner, 1968). Ps. aeruginosa may give rise to an acute infection but it frequently lingers in the lung in a state of chronic suppuration which is almost impossible to eradicate with antibiotics (Burns, 1973).

Cystic fibrosis (CF) is an inherited disease which results in the production of abnormal body secretions including a highly tenacious mucus in the lungs. This has the effect of creating a static condition in the lungs and consequently CF patients are very vulnerable to pulmonary infections. The staphylococcus is usually the primary pathogen but Ps. aeruginosa is a close contender and more difficult to treat. As a pseudomonas infection progresses in a CF lung, the organism tends to change to its mucoid form and this is associated with a concomitant decline in the clinical condition of the patient. The infections are of a chronic nature but despite massive pulmonary involvement, septicaemia rarely develops (Doggett and Harrison, 1972).

Babies, particularly premature babies, are susceptible to Ps. aeruginosa infection and their only predisposing debility may be their age and low natural resistance. Gastro-enteritis and diarrhoea caused by Ps. aeruginosa are found more frequently in children than adults and sometimes occur in epidemics in nurseries (Florman and Schifrin, 1950; Falcao et al., 1972). A maternity hospital was the scene of an outbreak of Ps. aeruginosa

infection which was unusual in that intestinal symptoms were mild or absent but four infants developed acute otitis media, four others conjunctivitis, and two mothers suffered from mastitis (Kwantes, 1960). Otitis media in babies, caused by Ps. aeruginosa, has also been reported by Carithers (1950) but in neither this nor Kwantes' report were any fatalities recorded. Otitis externa may develop from colonisation of the outer ear by Ps. aeruginosa, particularly in moist environments (Hall et al., 1968). Ear infections usually remain localised but occasionally Ps. aeruginosa invades deeper regions of the skull.

From this survey of Ps. aeruginosa infections, it is clear that the organism's versatility is not confined to its biochemical capabilities but embraces its ability to cause a wide variety of infections in compromised hosts.

Treatment of Pseudomonas aeruginosa infections

The problems of treating Ps. aeruginosa infections were realised soon after the pathogenic nature of the bacterium was recognised. Early forms of treatment were of two main types; directed either against surface infections or administered systemically. Prior to 1938 the agent used in the systemic treatment of Ps. aeruginosa infections was autogenous vaccine made from a culture of the organisms isolated from the lesion. Groves (1909) was probably one of the first to treat a patient with a Ps. aeruginosa infection with vaccine; following two injections of the vaccine the boy's pseudomonas pyaemia recovered.

Vaccine therapy was continued with variable results until the introduction of the sulphonamides (Freeman, 1916; Garretson and Cosgrove, 1927).

Taylor (1916) considered that Ps. aeruginosa was never a source of acute danger to a patient but caused delay in wound healing; an observation which has been frequently reported to the present day. Taylor noted that Ps. aeruginosa was less often present when discharges from wounds were acid, suggesting that treatment with an acid antiseptic might be efficacious. He found that of the acids he tested, 1% acetic acid in physiological saline was the most inhibitory to Ps. aeruginosa. Since this report, acetic acid has regularly been advocated for treatment of Ps. aeruginosa surface infections. Rank (1940) recommended the use of 2% acetic acid or household vinegar to prevent pseudomonas infection prior to skin-grafting, and more recent studies have confirmed the antipseudomonas activity of this acid (Hedberg and Miller, 1969; Rhoades and Short, 1970).

Vaccine therapy for Ps. aeruginosa infections fell into disuse with the advent of the sulphonamides in 1938 and later the antibiotics. Stanley (1947) reviewed some of the methods in use at the time and concluded that sulphonamides and streptomycin could be useful in treating Ps. aeruginosa infections but the sensitivity of the organism to these antibacterial agents *in vitro* was variable and strains that were initially sensitive to streptomycin sometimes became resistant during the course of treatment.

The innate resistance of Ps. aeruginosa to many antibiotics was coming to light and it was not until the introduction of

antibiotics directed specifically against the Gram-negative bacilli that much progress was made. The polymyxin group of antibiotics, particularly polymyxin B and E (colistin) emerged as one of the few groups of antibiotics that were active against systemic Ps. aeruginosa infections. The use of polymyxins has been reported more frequently from the U.S.A. where carbenicillin was not released for general use until recently. The problem with the polymyxins lies in their toxicity even at normal therapeutic doses. They are potentially nephrotoxic (Beirne et al., 1967) and can cause acute neuromuscular blockage leading to respiratory muscle paralysis in patients with poor renal function (Lindesmith et al., 1968).

Carbenicillin is a semi-synthetic penicillin with considerable potential for treating Ps. aeruginosa infections. It is also inhibitory to Proteus species and some strains of Escherichia coli but tends to encourage superinfection with resistant Klebsiella and Serratia organisms (Bodey, Rodriguez and Luce, 1969). Carbenicillin is inactive when administered orally but serum levels of 100 μg/ml can be achieved by giving large doses intravenously. There have not been any reports of serious toxicity even at the necessarily high doses. About 85% of Ps. aeruginosa strains from clinical isolates are sensitive to 100 μg/ml carbenicillin but much higher levels of the drug are required to inhibit the remaining 15% of strains (Washington, 1972). Bodey et al. (1969) reported the use of carbenicillin in 23 episodes of Ps. aeruginosa infection in cancer patients with a success rate of 91%. But even in the presence of high

serum levels of the antibiotic, bronchial and surface infections are often not eliminated. Carbenicillin treatment of Ps. aeruginosa pulmonary infections in children with cystic fibrosis sometimes results in clinical improvement but the organism is rarely eradicated from the sputum (Boxerbaum, Doeshuk and Pitman, 1968; Phair et al., 1968).

Gentamicin belongs to the amino-glycoside group of antibiotics and is active in vitro against a wide range of Gram-negative bacteria including Ps. aeruginosa, and some Gram-positive bacteria including neomycin-resistant staphylococci (Darrell and Waterworth, 1967). However, the antibiotic has been reported as nephrotoxic and causes injury to the vestibular division of the auditory nerve (Stone et al., 1965). Gentamicin has proved a useful addition to our armoury for attacking Ps. aeruginosa infections particularly in the topical treatment of burns, but patients with impaired defence mechanisms and granulocytopenia respond poorly to gentamicin therapy while carbenicillin treatment seems to be unaffected by these conditions (Rodriguez, Whitecar and Bodey, 1969).

Synergy has been demonstrated between carbenicillin and gentamicin in vitro at concentrations of each drug which are easily achieved in the blood. The mechanism of synergy may be due to carbenicillin inducing the formation of spheroplasts which are much more susceptible to gentamicin than are normal cells (Phair, Watanakunakorn and Bannister, 1969). This synergy may be useful in vivo but individual sensitivity testing of strains is necessary because a single combination of the two antibiotics

which is generally synergistic against a range of Ps. aeruginosa strains has not yet been found. Konicková and Prát (1971) found synergy between carbenicillin and gentamicin when the drugs were used in combination in the treatment of experimental Ps. aeruginosa pyelonephritis in rats, but these workers reported that the infection was only occasionally completely eliminated. Polymyxin B has also been reported as having some synergistic action with carbenicillin against Ps. aeruginosa (Phair et al., 1968).

However, it has recently been reported that carbenicillin is capable of inactivating gentamicin in vitro, and the half-life of gentamicin in the sera of eight end-stage renal patients was greatly reduced when carbenicillin was given concomitantly (Davies, Morgan and Anand, 1974).

Topical therapy

Surface infections caused by Ps. aeruginosa, especially those of burns, do not respond well to antibiotics administered parenterally and topical treatment is found to be better for reducing superficial infections and preventing invasion of the bacteria into the underlying tissues. As described earlier, antibacterial agents such as acetic acid were used in early years for topical treatment. In 1951, Jackson, Lowbury and Topley reported the value of local polymyxin treatment of Ps. aeruginosa in burns. Application of a 0.1% polymyxin-E cream greatly reduced the incidence of colonisation of burns by

Ps. aeruginosa. Polymyxin is safe for prolonged topical treatment because very little of the antibiotic is absorbed from wounds or mucous membranes.

The use of topically-applied gentamicin cream to reduce Ps. aeruginosa colonisation of burn wounds has proved very effective. In a water-miscible solution the antibiotic penetrates well and can reduce the numbers of Ps. aeruginosa to less than 10^5 per g of infected tissue (Stone, 1969). Uninterrupted treatment is necessary to prevent the emergence of resistant mutants but there is a tendency for Klebsiella and other resistant organisms to cause superinfections. The toxicity associated with the systemic use of gentamicin is much less serious when the drug is administered topically, but some workers feel that because of its effectiveness when given systemically, it should be witheld from topical use to discourage the development of resistance (Shuck, 1972).

Silver nitrate solution plays a prominent role in the local treatment of burns. Moyer et al. (1965) found that silver nitrate compresses were successful in reducing fluid loss from the surface of large burns and in preventing bacterial colonisation. Cason and Lowbury (1968) presented results which showed a significant reduction in the isolation of Ps. aeruginosa from burns and blood cultures after the introduction of local treatment with 0.5% silver nitrate compresses. Analysis of the data from one hospital showed that mortality due to burns had hardly changed over the period 1949-1964 but following the introduction of routine prophylaxis against Ps. aeruginosa with silver nitrate

compresses, there was a significant improvement in survival (Bull, 1971). However, silver nitrate can cause severe electrolyte depletion in a burned patient and occasionally methaemoglobinaemia develops.

Lindberg and his colleagues (1965) showed that the use of Sulfamylon (Mafenide) cream reduced the frequency with which Ps. aeruginosa was recovered from burns but there were some adverse side-effects. Lowbury and his co-workers (1971) carried out a controlled trial of the alternative forms of local treatment of burns, comparing compresses of silver nitrate with Sulfamylon cream and with exposure to warm dry air. They found that silver nitrate was generally more effective than Sulfamylon in maintaining the burns free from bacterial colonisation although there was little difference in the two forms of treatment against Ps. aeruginosa specifically. Both treatments were preferable to the exposure method. However Sulfamylon is very painful on application (Lowbury et al., 1971) and has sensitizing properties which may lead to long-lasting cross-sensitization to other sulphonamides (Bleumink and Klokke, 1971). It is also a potent inhibitor of carbonic anhydrase and causes respiratory and metabolic complications. Thus both silver nitrate and Sulfamylon have disadvantages as agents for topical therapy.

Silver sulphadiazine is a compound designed to combine the antibacterial activities of silver and sulphadiazine. It is active against Ps. aeruginosa and some strains of Proteus and Staphylococcus aureus (Fox, Rappole and Stanford, 1969).

Disadvantages in its use have not been reported to date and it may have an important role to play in the treatment of burn infections.

Resistance of Pseudomonas aeruginosa to antimicrobial agents

Ps. aeruginosa has a diversity of enzyme systems which allow it to grow on many different substrates. Therefore it is difficult to interfere with an essential enzyme pathway and this may partly account for the organism's natural resistance to many antimicrobial agents. Treatment of Ps. aeruginosa infections is further complicated by the acquisition of resistance by sensitive strains.

Some forms of resistance in Ps. aeruginosa are thought to be associated with changes in the cell wall structure. For example, cells which are resistant to polymyxin have much less phospholipid in their cell walls than sensitive cells. Phospholipid is thought to be involved in the passage of polymyxin to its active site at the cytoplasmic membrane where it destroys the osmotic barrier activity (Lindesmith et al., 1968), and if the phospholipid is reduced, much higher levels of antibiotic are required in order that some should reach its target (Brown and Watkins, 1970).

Resistant mutants of Ps. aeruginosa can be isolated from cultures grown in the presence of sub-inhibitory concentrations of carbenicillin. These carbenicillin-resistant cells are thought to have changes in the type or amount of cell wall lipopolysaccharide (Barrett and Asscher, 1972). The margin between the concentration of carbenicillin obtainable in the serum and the minimal inhibitory dose of many clinical isolates of

Ps. aeruginosa is small but much higher concentrations of the antibiotic can be obtained in the urine. Thus Ps. aeruginosa is usually rapidly eradicated from the urinary tract by carbenicillin therapy but may persist in extraurinary infections. Holmes and his colleagues (1969) found that persistent Ps. aeruginosa increased their resistance to carbenicillin fourfold or more during the course of treatment. Resistance to carbenicillin does not only arise from selection of resistant mutants but also by acquisition of resistance determinants on resistance transfer factors. In the Birmingham Accident Hospital, Lowbury and his colleagues (1969) reported a gradual increase in the frequency of isolation of carbenicillin-resistant strains of Ps. aeruginosa over the period 1966-1968, but early in 1969 highly resistant strains began to appear and quickly ousted the more sensitive strains of Ps. aeruginosa. Resistance appeared to be due to the production of a carbenicillinase enzyme and a resistance transfer system was implicated (Lowbury et al., 1969). Later an R factor was isolated from these resistant strains and was found to confer on the cells resistance to carbenicillin, neomycin, kanamycin and tetracycline (Grinsted et al., 1972). The R factor was shown to be a plasmid which was freely transmissible between strains of Ps. aeruginosa, Esch. coli and Proteus mirabilis in vitro and in vivo in experimental infections of mouse burns (Roe, Jones and Lowbury, 1971).

Resistance to gentamicin has also been observed to develop during the course of treatment and may be encouraged by the rapid fall-off in serum levels of the drug leaving sub-inhibitory

concentrations (Darrell and Waterworth, 1967).

Thus we are thwarted in our treatment of Ps. aeruginosa infections both by the innate resistance of the organism to many antibiotics and by its ability to acquire resistance to the few effective antibiotics such as carbenicillin. With these problems in mind, earlier forms of treatment have been reconsidered, particularly vaccine therapy.

Immunotherapy

Treatment of Ps. aeruginosa infections by vaccination is not a new idea and Groves (1909) used a vaccine to treat a pseudomonas pyaemia. But early vaccine therapy had variable results and was later superseded by antibiotic treatment. In recent years the failure of antibiotics to cure Ps. aeruginosa infections, for reasons outlined above, has led to a revival of interest in vaccines. Work in this field has focussed particularly on Ps. aeruginosa infections of burns which often have fatal consequences. Although burned patients are more susceptible to Ps. aeruginosa infections than normal people, their antibody production against chemical and particulate antibodies is normal and thus active immunisation is a feasible proposition.

Various fractions of Ps. aeruginosa cells have been shown to be antigenic in animals, for example the slime (Liu et al., 1961), the LPS of the cell wall (Young, 1972), and the extracellular enzyme fraction (Jones, 1968). It is thought that the LPS

constitutes the principal antigenic component (Young, 1972). Vaccines can be prepared using whole cells and these stimulate good protection in animals against infection by a homologous strain but the protection is less good against a heterologous strain (Liu et al., 1961). Fisher, Devlin and Gnabasik (1969) established an immunotype scheme based on protective antigens. They prepared whole-cell killed vaccines from 342 strains of Ps. aeruginosa from clinical sources and tested the ability of each vaccine to protect mice against infection by each of the other strains. Thus these workers were able to group their strains on the basis of cross-protection. Seven immunotypes emerged and from these a heptavalent vaccine was prepared. In 1971, Alexander, Fisher and MacMillan reported the success of the clinical trials of this vaccine in burned patients. When a suitable dosage was given, the vaccine could elicit a rapid antibody response which was capable of being sustained and which was associated with protection of the patient against subsequent infection by Ps. aeruginosa. The results of Alexander et al. (1971) show a significant reduction in mortality from pseudomonas sepsis among seriously burned patients but they recorded the presence of side-effects to the vaccine in some of their patients probably due to the LPS of the purified antigens which are used to prepare the vaccine. However the side-reactions were considered insignificant in relation to the danger of an infected burn.

Active immunisation involves a time-lag of about 5 days between administration of the antigen and maximum antibody

production and during this time the patient remains unprotected against Ps. aeruginosa. However, it has been shown that the patient may be protected against infection by passive immunisation with hyperimmune serum (Jones, 1968; Pierson and Feller, 1970).

The recent success of vaccine therapy suggests that this form of treatment may be useful in protecting other groups of patients who are highly susceptible to Ps. aeruginosa infections but whose antibody production is normal. But Ps. aeruginosa infections are also common sequelae of primary debilitating diseases in which antibody production is naturally or artificially depressed and there are still considerable problems associated with the treatment of Ps. aeruginosa infections in such patients.

CHAPTER II

PYOCINES

Like other forms of life, bacteria are subject to infection and inhibition by a range of viruses and viruslike particles which fall naturally into two main categories: bacteriophages and bacteriocines. The first descriptions of these two types of inhibitory agent were made within ten years of each other. In 1915, Twort isolated a virus responsible for the lysis of staphylococci, and in an independent study in 1917, d'Herelle described the dysentery bacteriophage. d'Herelle coined the term "bacteriophage" (literally "eater of bacteria") which is now commonly abbreviated to phage. A few years later, Gratia (1925) recognised the presence of an inhibitory agent in the culture filtrate of Escherichia coli V which acted specifically against Esch. coli β. This agent showed many similarities to phage but was unable to reproduce itself in the sensitive cell which it attacked and was later defined as a bacteriocine. During the next 20 years there was little published work on the bacteriocines, but by 1946, Gratia and Fredericq had examined a number of strains of Esch. coli and found that the inhibitory phenomenon was fairly general. They named the agents responsible, colicines. Although recognising that the activity of colicines was highly specific, directed against other strains of the same genus, they found that some strains of Shigella and Salmonella were also susceptible.

By 1948, Fredericq had recognised 17 colicines that were

distinguishable by their different spectra of activity, the morphology of their inhibition zones, the extent of their diffusion through agar, their ability to pass through cellophane and their sensitivity to heat and proteolytic enzymes. Fredericq suggested that cells that were sensitive to attack by more than one colicine possessed specific receptor sites for each colicine. He also found that colicine-resistant mutants arose spontaneously from the parent culture and were stable. He used this latter feature in a classification scheme for colicines based on the specificity of resistant mutants.

As Esch. coli has played a key role in microbial genetics, much of the early work on bacteriocines centred on colicines. However, Pseudomonas aeruginosa is one of the oldest known examples of a bacterium that produces substances that are antagonistic to other bacteria. Bouchard (1889) was the first to recognise the antibiotic activity of preparations of Ps. aeruginosa against Bacillus anthracis and later this agent was marketed as a commercial product, pyocyanase, and was used in the treatment of diphtheria, grippe and meningitis; however, it fell into disrepute because the manufacturers "failed to produce a potent product" (Hays et al., 1945). These early studies did not examine the antagonistic activity of culture filtrates of Ps. aeruginosa against other strains of the same species and it was not until 1954 that bacteriocine activity was recognised among Ps. aeruginosa strains and the term pyocine was introduced (Jacob, 1954). It is the specific activity of pyocines against other strains of Pseudomonas that separates them from other

antibiotic substances produced by Ps. aeruginosa. Prior to his description of pyocines in 1954, Jacob and Wollman (1953) had proposed the name bacteriocine for

> "these particular antibiotics which appear to be protein-like substances, the synthesis of which leads to bacterial lysis, and are adsorbed on specific bacterial receptors."

Jacob realised that bacteriocine activity was probably not confined to colicines and pyocines and it is now appreciated that bacteriocine production is a widespread phenomenon. Bacteriocines have been recognised in the Gram-positive genera Staphylococcus (Fredericq, 1946), Streptococcus (Kelstrup and Gibbons, 1969), Lactobacillus (Upreti and Hinsdill, 1972) and Bacillus megaterium (Holland, 1962). Among the Gram-negative organisms, bacteriocine activity has been identified in Vibrio cholerae (Lang, McDonald and Gardner, 1968), Neisseria gonorrhoea (Flynn and McEntegart, 1972), Serratia marcescens (Foulds, 1971), Enterobacter cloacae (de Graaf et al., 1968) as well as in Shigella and Proteus species, and this list is by no means exhaustive.

The nomenclature of bacteriocines is based on the specific name of the producing organism, e.g. pyocine is derived from Ps. pyocyanea and although the organism is now more frequently known as Ps. aeruginosa, the term aeruginocin, which has been suggested, seems rather unwieldy.

The class of agents referred to as bacteriocines includes a wide range of substances from simple enzymes (e.g. megacin A is a phospholipase) to complex particles resembling viruses (e.g. some

pyocines and a vibriocin). A sub-division of the heterogeneous collection of substances can usefully be made into two main groups; the high molecular weight bacteriocines, which appear to be of viral origin or have viral associations, and the low molecular weight agents, which appear to be constituents of the bacterial cell (e.g. associated with the somatic antigen). According to Bradley (1967), these two groups probably have quite separate origins and occupy different positions on the evolutionary scale. It is frequently found that each family of bacteriocines, for example the pyocines or the colicines, consists of representatives of the two different groups. Sandoval, Reilly and Tandler (1965) postulated that if colicines were the products of defective lysogeny, they may range from relatively simple proteins to completely formed phage particles. All the bacteriocines that have been studied in sufficient detail have been shown to include or consist of protein or polypeptide and do not have any DNA or RNA.

Hamon (1956) extended Jacob's work and examined 15 strains of Ps. aeruginosa of which ten produced pyocines. He defined some of the properties of pyocines: inactivated by heating, non-dialysable, diffusible in agar, sometimes sensitive to proteolytic enzymes, possess antigenic properties, resistant to DNase and ultra-violet irradiation, precipitated by ammonium sulphate and by acetone. Hamon described the pyocines as "lethal corpuscles of protein nature."

Physical and chemical properties of pyocines

Among the bacteriocines produced by Ps. aeruginosa, a variety of agents are recognisable. There are low molecular weight pyocines, frequently associated with the cell wall, especially the lipopolysaccharide (LPS) of the somatic antigen. A cell wall-associated protein with pyocine activity was described by Homma and Suzuki (1964), and Homma, Goto and Shionoya (1967) identified pyocine A_3 from the cytoplasm and pyocine A_2 from the cell wall of Ps. aeruginosa strain PI-III. The molecular weight of A_2 was estimated as 23,000. These low molecular weight pyocines are known as S-type or small. Ito, Kageyama and Egami (1970) found small-pyocine activity in the supernates of strains of Ps. aeruginosa M11 and P28 and estimated the molecular weight of the agents to be c. 100,000. Recently, a detailed study of a small pyocine was described (Ohkawa, Kageyama and Egami, 1973). Pyocine S2 was produced by strain M47 and appeared to be a simple protein of molecular weight c. 7.5×10^4, and thus of the same order as some colicines (E_1, E_2, E_3, I_a, I_b, and K).

The pyocines that have been studied more extensively are those that belong to the high molecular weight group (1×10^6 - 1×10^7). The morphological similarity of these pyocines to the tails of T-even coliphages has frequently been observed and indeed some authors believe that these substances should be regarded as defective phages and should not be defined as bacteriocines (Reeves, 1972).

Pyocine R was one of the first pyocines to be visualised in

the electron microscope and was described by Ishii, Nishi and Egami (1965) as being composed of rod-shaped contractile structures. The original pyocine, C10, of Jacob (1954) was examined by Bradley (1967) and found to be morphologically identical to pyocine R. In their earlier paper, Higerd, Baechler and Berk (1967) could only recognise contracted particles in electronmicrographs of pyocine C9 but suggested that this might have been an artefact of the preparation. Later, they described contracted and relaxed forms of the pyocine (Higerd, Baechler and Berk, 1969) and suggested that the uncontracted state was the active form. Higerd and his colleagues (1969) examined pyocine preparations after various chemical treatments and found that contraction of the sheath was promoted by 0.5M-$MgCl_2$, 1% formalin, low pH, sonic treatment, and freezing and thawing. The contraction brought about by 0.5M-$MgCl_2$ could be reversed by reducing the salt concentration to 0.02M-$MgCl_2$.

Yui (1971) examined pyocine R in detail and suggested that it was composed of at least four structural components: sheath, core, baseplate and fibres. It is the sheath which is capable of contraction. Govan (1974a) examined several pyocines in the electron microscope and distinguished the same components. He found that in the relaxed state, the pyocine particles possess a helical or horizontally striated substructure of about 20 subunits and measure c.100 x 15nm. In the contracted particles, a hollow core can be seen, partially enclosed in the sheath. The amino acid analysis of the components (Yui, 1971) showed that the sheath, which occupies 53% of the pyocine particle, did not vary greatly

from the other parts and when compared with the amino acid composition of coliphage T2, the pyocine and phage had a common feature in the high amounts of hydrophobic amino acids. Treatments that dissociate the sheath into its subunits suggest that it has an extraordinarily rigid quaternary structure.

The activity of pyocine R is inhibited by p-chloromercuribenzoate and mercuric chloride (Kageyama and Egami, 1962) suggesting that free sulphydryl groups exist in the pyocine which are responsible for its activity. However, the results are inconclusive because other sulphydryl agents did not affect pyocine activity (Yui, Ishii and Egami, 1969). The features of the pyocine structure that are responsible for its activity are as yet undetermined but contraction of the sheath seems to play an important role (Govan, 1974a). However, pyocines have been described that are lacking this contractile sheath. In 1967, Takeya et al. produced electronmicrographs of pyocine 28, which appears as a rod-shaped particle of width c.9nm and of variable length (50 - 400nm). The fibrous shape and the width of the particle resemble the pseudomonas phage Pf (Takeya et al., 1969). The specific attachment of pyocine 28 to sensitive cells could be observed in the electronmicrographs. Since the discovery of the filamentous form of pyocine particle, a number of others of the same nature have been described (Brown, 1973; Govan, 1974b).

Although both classes of high molecular weight pyocines are considered to be proteins, their activity is unaffected by proteolytic enzymes such as trypsin, chymotrypsin and pronase. The pyocines are also resistant to DNase, and RNase, 10^{-3} M-cyanide,

10^{-3} M-ethylenediaminetetraacetic acid (EDTA), 10^{-3} M-cysteine and 0.1 - 0.7M-NaCl. The activity of the contractile pyocine is unaffected by heat below 70°C, but at or above 70°C, the pyocine is rendered inactive within 10 min. (Higerd et al., 1967). The filamentous pyocine is more heat-labile, being almost completely inactivated by heating at 60°C for 10 min. (Takeya et al., 1967).

The small (S-type) pyocines appear to constitute a distinct category of substances. Although they were recognised fairly early in pyocine history, fewer detailed studies have been published. The small pyocines are sensitive to the action of proteolytic enzymes and are considerably more heat-labile than the high molecular weight particles. Homma and Shionoya (1967) suggested that the small pyocines A2 and A3 might be included in the structure of high molecular weight particles and it is only when they remain unprotected that they are sensitive to proteolytic enzymes. The study of pyocine S2 by Ohkawa et al. (1973) revealed that it was an apparently simple protein, whose amino acid composition had characteristics in common with certain colicines; rich in charged and polar amino acids and lacking in cysteine residues. The lack of sulphydryl groups in small pyocines is in contrast to contractile pyocines where it is thought that these groups may play a role in the activity of the particles. The small pyocines are non-sedimentable and have not been resolved in the electron microscope. They would appear to be similar in nature to colicines.

Production of pyocines

The production of pyocines by pyocinogenic cells is dependent on the cultural conditions; Govan (1968) found that 32°C was the optimum temperature for pyocine production. A few cells in the population will synthesise pyocine spontaneously but production by the majority of cells can be induced by treatment of a culture with mutagenic agents such as ultra-violet irradiation or Mitomycin C. After induction, the turbidity of the culture rises and then falls sharply as the cells lyse and the pyocine is released.

The mechanism of induction is not clearly understood. The lethal effects of ultra-violet irradiation on a cell are thought to be due to the formation of pyrimidine dimers in the DNA. In the presence of bound acriflavine, bacteria are protected against the lethal effects of ultra-violet because pyrimidine dimerisation is inhibited, but acriflavine does not protect against induction of pyocine production, which suggests that induction is not caused by pyrimidine dimersation (Alper, Forage and Hodgkins, 1972).

Mitomycin C is an antibiotic produced by Streptomyces caespitosus whose primary effect is the inhibition of DNA synthesis. In the cell, the antibiotic is converted to an activated form which covalently bonds with the DNA, leading to the formation of cross-links between complementary DNA strands (Waring, 1966). The biological effects of Mitomycin C and ultra-violet irradiation show distinct similarities.

Experiments using radioactively-labelled sulphur showed that

the contractile pyocine R is synthesised de novo after induction and there is little or no transfer of host protein to the pyocine (Ikeda, Kageyama and Egami, 1964). Similar experiments have not been reported for small pyocines, but if these form part of the cell wall protein or other normal cell components, then their synthesis might be expected to differ from that of high molecular weight pyocines. Following induction of the pyocine R producer strain by ultra-violet irradiation or Mitomycin C, the pyocine proteins appear after 10 - 15 min. and the active pyocine particles after about 25 min. Electronmicrographs taken 45 min. after induction show large numbers of pyocine particles in cell extracts (Shinomiya, 1972). The killing activity of pyocine R appears several minutes after the emergence of antigenic pyocine protein, suggesting that this interval is the time required for the assembly of the subunits. The pyocine particles have no intrinsic antibacterial activity and must be assembled to produce active particles (Shinomiya, 1972). DNA synthesis in the producer cell is greatly reduced after induction, and treatment of an induced culture with an inhibitor of DNA synthesis (5-fluorodeoxyuridine) has little effect on pyocine production (Shinomiya and Egami, 1967). There is little change in RNA synthesis after induction and pyocine protein accounts for about 30% of the total protein synthesis.

Pyocine R production can be inhibited by addition of the amino acid analogue fluorophenylalanine (FPA) (Ikeda, 1967) and by dihydrostreptomycin (DHSM) (Shinomiya and Egami, 1967). Both agents are specific inhibitors of pyocine synthesis and other protein synthesis continues unaffected. DHSM is thought to act

through specific effects on ribosome structure, but the mechanism of FPA inhibition is not understood.

The mode of action of pyocines

It is difficult to generalise on the mode of action of pyocines because few of these agents have been examined in sufficient detail. Analogies have been drawn with the action of colicines which have been studied in greater depth. The general picture of the mode of action of bacteriocines suggests that they are bactericidal rather than bacteristatic and that a single bacteriocine molecule, adsorbed to a specific receptor site on the sensitive cell surface, is capable of killing that cell. The specific biochemical mechanism of the host cell that is affected varies with the attacking bacteriocine. However, the inhibitory activity is clearly different from that of classical antibiotics which enter the cell and inhibit enzyme function or in some cases bind to DNA.

Jacob (1954) showed that after addition of pyocine C10 to a sensitive bacterial culture, the cells did not multiply and their respiration gradually decreased. A similar result has been found with colicine K. Kageyama, Ikeda and Egami (1964) studied the mode of action of the contractile pyocine R. At 37°C, adsorption was followed by release of ultra-violet-absorbing material (at 260nm) and death of the cell. Under defined conditions, the amount of ultra-violet-absorbing material released is proportional to the dose of pyocine added. If a large excess of pyocine is added, the cells lyse, possibly due to the presence of a lytic

enzyme. Kageyama et al. (1964) found that a muramidase-like enzyme is synthesised at the time of induction of a pyocinogenic strain and aids in the release of pyocine from the producer cell. At this stage, a certain portion of the enzyme becomes incorporated into the pyocine structure. An analogous situation has been reported in some phages.

Kaziro and Tanaka (1965a) studied the mode of action of pyocine R and found that addition of the pyocine to a sensitive cell culture resulted in immediate and complete inhibition of DNA, RNA and protein synthesis. They postulated that the cessation of protein synthesis was due to inactivation of the cell's ribosomes (Kaziro and Tanaka, 1965b).

If single-hit kinetics operate, i.e. one pyocine particle is capable of killing a single cell, then the survival of a sensitive cell population can be calculated from a Poisson distribution. The results suggest that a straight line relationship exists between the logarithm of the survival fraction and the dose of pyocine. Kageyama et al. (1964) found that this relationship held true but there were inconsistencies at high concentrations of pyocine. They suggested that this might be due to the high frequency of mutation to pyocine resistance. If one pyocine particle is capable of killing one bacterial cell, from a killing experiment it is possible to calculate the number of pyocine particles present and hence the number produced by a pyocinogenic cell. Thus, Kageyama and his colleagues (1964) estimated that one cell was capable of elaborating about 200

pyocine particles.

Ohkawa et al. (1973) found that the small pyocine S2 also showed a single-hit killing process. The amount of protein corresponding to one killing unit was calculated as c.3.7×10^{-11} µg or about 300 molecules. A similar result was obtained by Nomura and Maeda (1965) for colicine E2.

Several theories have been devised to try and explain the mode of action of colicines and although there has been no counterpart in pyocine studies, it is interesting to examine the colicine theories in view of the similarities between these agents and pyocines. Nomura (1967) proposed a model for the mode of action of colicines based on the following four points:

(1) attachment of the colicine to a specific receptor site on the cell surface

(2) reversible change in the cell attachment site

(3) transmission of the change to the "target"

(4) effects on biochemical and killing target

The important feature of the model was the idea that a mechanism that conveys the stimulus generated by the colicine at its receptor site to a biochemical target involves an action transmitted through the cytoplasmic membrane. Changeux and Thiery (1967) postulated a method by which such a change might be effected in the cytoplasmic membrane. They suggested that colicines are specific components of the cell membrane of colicinogenic strains that possess a high affinity for the cell surface of sensitive strains. The bacterial cell membrane is viewed as a co-operative structure

composed of repeating units arranged in a two-dimensional lattice. At least two structural conformations are available to the units within the lattice and the transition of one unit from one state to another tends to affect its nearest neighbour to change in the same manner. The binding of a colicine molecule to the membrane induces the transition of a small number of membrane units to a conformational state which is not usually present in large amounts in the resting cell. The transition tends to spread among the membrane units until the whole cell membrane is shifted to this unusual conformation. The biochemical changes induced in the cell by colicine attack are the results of changes in the conformation of the proteins that are attached to the inside of the cell membrane. The biological activity of these proteins is consequently upset and the cell is unable to function.

This theory of Changeux and Thiery (1967) assumes that the colicine molecules bind to the cell membrane, but more recent work has shown that the bulk of colicine receptor activity is located in the cell wall (Sabet and Schnaitman, 1973). How the colicines penetrate the cell surface is at present one of the most intriguing aspects of colicine action. The fact that pyocine receptors also appear to be located in the cell wall suggests another similarity between the mode of action of these two groups of bacteriocines.

The complex pattern of production of and sensitivity to pyocines in Ps. aeruginosa suggests that at least some strains are

capable of producing more than one pyocine and that sensitive cells possess a number of different receptor sites. In general, bacteriocinogenic cells are insensitive to the action of the bacteriocines they produce, but Goodwin, Levin and Doggett (1972), examining mucoid and non-mucoid strains of Ps. aeruginosa, suggested that some strains were autosensitive. A similar finding has been reported for colicines but the results are somewhat dubious since the immunity of cells to the colicine they produce is a primary definition of colicine classification (Bowman, Sidikaro and Nomura, 1973).

The specific adsorption of pyocines to sensitive cells was described by Hamon and Peron in 1960 and later Ikeda and Egami (1969) extracted the receptor substance for pyocine R. The latter authors found that receptor activity could not be attributed to a single homogeneous substance, but was associated largely with the cell wall LPS. The examination of a similar extract from a cell that was resistant to pyocine R showed that it behaved very differently. The resistant strain was also resistant to pyocine R3 and R4 and to phage PS5. More recent work by Ikeda and Egami (1973) confirmed that the receptor for pyocine R is composed of LPS and the size of the site required for receptor activity was calculated as $10^5 - 10^6$. Stewart and Young (1971) also confirmed that lipid materials, particularly in a complexed form, play an important role in receptor activity for contractile pyocines. Treatment of cells with EDTA resulted in a considerable reduction in pyocine-adsorbing power. EDTA has been shown to chelate divalent cations which are essential to the structural integrity

of the LPS in the cell wall. Further evidence for the role of LPS as the receptor material for contractile pyocines was provided by electronmicrographs showing the contractile pyocines 21 and 430 adsorbed to LPS extracted from a sensitive indicator strain (Govan, 1974a).

The receptor sites for filamentous and small pyocines have not been described but Govan (1974b) demonstrated in electron-micrographs that filamentous pyocines attached to extracted LPS, but in neutralisation experiments, the LPS failed to neutralise the pyocine activity.

The genetics of pyocinogeny

The ability to produce bacteriocines is a heritable characteristic, which may be transferable from one cell to another. The genetic information for colicine production is thought to be borne on plasmids known as Col factors. Little is known about the genetics of pyocinogeny, partly because experimental genetic systems are less well-established in *Ps. aeruginosa* than in *Esch. coli*.

Kageyama (1970a) showed that the genetic determinant for pyocine R2 could be transferred from a donor cell to a recipient by conjugation and that it was closely linked to the tryptophan and other chromosomal markers. This suggests a chromosomal location for the pyocine determinant. Pyocine R2 is a contractile pyocine and thus may be likened to a defective phage. The chromosomal position of its determinant may correspond to the integration site of certain prophages (Kageyama, 1970a). Using

the general transducing phage F116, Kageyama (1970b) also showed that the genetic marker for pyocine R2 could be transduced into recipient cells and again there was a high degree of linkage between the pyocine and trytophan markers.

More recently, Kageyama, Shinomiya and Ohsumi (1973) have reported that the genetic determinants for pyocines R and R3 can also be transferred to a recipient strain by conjugation and transduction.

As yet, nothing is known about the genetics of filamentous and small pyocines. If the latter are truly to be likened to colicines, one might expect their genetic information to be plasmid-borne.

Relationships between bacteriocines and bacteriophages

Since the earliest descriptions of bacteriocines, their likeness to bacteriophages has been commented on repeatedly. The two types of inhibitory agent have in common: similar action spectra; fixation on to specific receptors; specific immunity to the agent produced; biosynthesis that is both inducible and lethal. The major difference between bacteriocines and phages is that the latter are self-reproducible. Despite the similarities between the two types of agent, the actual relationship is somewhat obscure. Bacteriocines resemble virulent phage in their killing action, but bacteriocinogenic factors resemble lysogenic (temperate) phages, which are thought to be incapable of killing sensitive bacteria.

The obvious similarity between contractile pyocines and the tails of the T-even phages suggests that one may have evolved from the other. According to Bradley (1967) it seems unlikely that a cell would develop the genetic information to code for a selection and penetration unit before providing itself with a suitable receptacle for the transmission of genetic information, i.e. the phage head. Thus it seems possible that contractile particles may have developed as a result of the loss from a temperate phage genome of the ability to code for a head. Contractile pyocines have been likened to phage ghosts, which are capable of attaching to and killing sensitive cells, but differ from their parent phage in being unable to replicate (Herriot and Barlow, 1957).

The filamentous pyocines, of which pyocine 28 is an example, are thought to be related to fibrous pseudomonas phages, but structures resembling pyocine 28 could not be produced from Pf phage particles by various physical and chemical treatments (Takeya et al., 1969). Pyocine 28 also resembles the core section of a contractile type of pyocine but the filamentous pyocine is often curved, whereas the cores are always straight. Bradley (1967) suggested that the fine structure of pyocine 28 showed close similarities to the long sheathless tails of pseudomonas phages of group B. Whatever the correct comparison is, it seems reasonable to consider that both the high molecular weight types of pyocine may have evolved from defective phages.

The low molecular weight bacteriocines appear to be quite

different from their phage-like, high molecular weight counterparts. It has been postulated that the small bacteriocines may be related to fertility (F) factors (Reeves, 1965; Bradley, 1967). Since the determinants for colicines (col factors) are thought to be plasmid by nature, a possible side-evolution from the F factor is suggested. However, this is conjecture and the true origin of these agents and the relationship of the small pyocines to normal cell wall components is not understood.

The serological activity of pyocines

Pyocines are good antigens and immune sera can easily be produced in animals. Kageyama et al. (1964) demonstrated a precipitation reaction between pyocine R and antiserum. A 1 in 100 dilution of serum was sufficient to neutralise pyocine of 10,000 units activity. A number of workers have examined the serological relationships between different pyocines and between some pyocines and phages. Homma et al. (1967) found that antisera produced against each of the contractile pyocines Al_{mc}, R_{sp}, and R_{mc} neutralised the activity of all three. The activity of pyocine Al_{mc} could also be neutralised by antisera raised against five temperate phages produced by the pyocinogenic strain. Phage PS3 is similar to, but not identical with, the R-type pyocines and has similar receptor specificity. Antiserum against pyocine R was capable of neutralising the phage activity but antiserum produced specifically against the pyocine sheath protein did not neutralise phage activity. This suggests that

the pyocine component which is common to the phage resides elsewhere than in the sheath (Ito and Kageyama, 1970). Homma et al. (1967) found that the activity of pyocine 28 was neutralised by the same antisera raised against contractile pyocines Al_{mc}, R_{sp}, and R_{mc} and the five temperate phages. Serologically, pyocine 28 was the same as pyocine Al_{mc}, but these workers did not confirm that pyocine 28 had a filamentous form (Takeya et al., 1967). Strain 28 has been reported to produce other types of pyocine as well as the filamentous variety (Ito et al., 1970). Govan (1974b) found that in strain 430, which produces both contractile and filamentous pyocines, no serological relationship could be demonstrated between the two types of pyocine particle.

The small pyocines A2 and A3 showed a serological similarity with each other, while being quite distinct from the high molecular weight pyocines. Antisera produced against the whole bacterial cell or against the cell wall LPS did not neutralise the pyocines A2 and A3 despite the close association between pyocine A2 and the cell wall (Homma and Shionoya, 1967).

The uses of bacteriocines

The biological significance of bacteriocines in nature is probably that of conferring a selective advantage to the bacteriocinogenic strain, despite the actual synthesis of bacteriocine being lethal to the producer cell. Production of bacteriocine by a few cells in a clone may, by killing the

surrounding bacteria of the same or related species, improve the prospects of the clone. The existence of bacteriocines will tend to lead to selection for loss of receptor sites on sensitive cells (Reeves, 1965). Thus bacteriocines in their natural habitat may influence the evolution of bacteria.

It has been suggested that bacteriocinogenic strains may have a selective advantage in colonising areas such as the human gut. Colicine production has been thought by some workers to be a likely mechanism for the control of the intestinal flora, but the results are variable. Hentges and Freter (1962) examined the antagonism of intestinal bacteria against Shigella flexneri in vitro and in vivo and they concluded that colicine-like substances had no role to play. They also pointed out the problems of relating in-vitro findings to the in-vivo situation. Branche and his colleagues (1963) studied Esch. coli in normal healthy humans over a period of six months and assayed the strains isolated for colicine production against a single indicator strain. These workers concluded that the resident serotypes of Esch. coli in the gut produced colicine in vitro more consistently than did the transient flora. Thus colicinogeny appeared to be an advantage to a strain wishing to establish itself in the gut. The argument for and against colicine production as a selective advantage has continued over the years. Braude and Siemienski (1968) claimed that in double urinary infections in rats with a colicinogenic and a colicine-sensitive strain, the latter was eradicated in about one quarter of the infected animals. But Ikari, Kenton and Young (1969), examining the interaction between colicinogenic and

colicine-sensitive strains in germfree mouse intestine, concluded that colicine production itself did not contribute significantly, if at all, in the competition for dominance in the gut.

All the studies of the selective advantage of bacteriocine production seem to have been carried out with respect to colicines and there have been no reports of similar studies with pyocines.

The practical use of bacteriocines as tools by man is more tangible. The prime importance of pyocines particularly, has been in the development of epidemiological typing systems. Bacteriocine typing originated with colicine typing, developed by Abbott and Shannon (1958) and later modified by Gillies (1964) to type strains of Shigella sonnei. This method relies on colicine production by the unknown strain as indicated by the sensitivity of a standard set of indicator strains. Sensitivity of the unknown strain to a stock set of colicines was also examined by Abbott and Shannon but was found to be unreliable and was not pursued. During the last fifteen years, bacteriocine typing has blossomed and has been applied to various genera with variable results. Pyocine typing of strains of Ps. aeruginosa has probably emerged as the most widely used and the most reliable of the bacteriocine typing techniques.

In 1960, Holloway suggested that since about 90% of Ps. aeruginosa strains are capable of producing pyocines, typing by production seemed a feasible proposition. Within four years, Darrell and Wahba (1964) published a pyocine typing technique

that allowed the recognition of 12 distinct types. They reported that pyocine production was a stable characteristic; an important feature of an epidemiological marker. Whereas Darrell and Wahba relied on pyocine production by the unknown strain, Osman (1965) described a method of pyocine typing based on the sensitivity of the unknown isolate to four standard pyocine preparations. By this method, he was able to differentiate 101 strains into 10 types.

In 1966, Gillies and Govan published their pyocine typing technique based on production of pyocines by the unknown strain of Ps. aeruginosa. After examining the effect of medium, temperature and time of incubation on pyocine production, they concluded that temperature was critical and the optimum condition for pyocine production was 32°C. Complex media were not required and the method was attractive because of its simplicity. Using eight standard indicator strains, 36 pyocine types were recognisable. However, as many as 30% of strains belong to pyocine type 1, thus reducing the usefulness of the method for discriminating between strains. By introducing a further set of five sub-typing indicators, Govan and Gillies (1969) were able to subdivide type 1 strains into eight sub-groups. The validity of the technique was confirmed and the method is now used in many centres.

Other methods of pyocine typing have been described (Farmer and Herman, 1969) and some of these combine pyocine and serological typing in an effort to obtain finer subdivision of strains. Siem (1972) found the differentiation between strains was better when serological and pyocine typing were combined than with either

method alone. He also found that there was a distinct relationship between the pyocine types and the serotypes, which to some extent reduced the differentiation. Pyocine typing does not enjoy universal acceptance in the same way as staphylococcal phage typing, partly because phage and serological typing techniques are also available for Ps. aeruginosa. All three methods have their disadvantages but pyocines certainly have a useful role to play.

Another use of pyocines that has been recognised more recently is that of a tool in bacterial genetics. Nomura's model of bacteriocine action (1967) proposed that the specific target for the action of all bacteriocines was at the cell membrane of the sensitive cell. He predicted that it should be possible to isolate mutants that could adsorb bacteriocine to the cell surface but were not killed. Such mutants can be isolated from strains of Ps. aeruginosa by mixing the cells with an excess of a small pyocine (Holloway, 1971). Two types of resistant mutant result; those cells that have an altered surface and are unable to adsorb the pyocine, and those that adsorb pyocine but are insensitive to its action. The latter group are referred to as tolerant mutants. The genetic change in tolerant mutants involves loci that are involved in cell membrane synthesis, and in addition to pyocine resistance, tolerant cells also show a range of pleiotropic effects. These include changes in membrane permeability and in various DNA functions, an increased sensitivity to ultra-violet irradiation and a decreased ability to undergo genetic recombination. Changes may also occur in the functions

of enzymes associated with the membrane. Thus the small pyocines have provided a useful mechanism for isolating membrane mutants from which we hope to learn more about the structure and function of the bacterial cell membrane.

The use of pyocines as therapeutic agents

The inhibitory activity of bacteriophages and bacteriocines has suggested the possible development of these agents for therapeutic use. Phages were used for treating cholera for a number of years with variable success. In 1929, a programme of phage treatment, combined with anti-cholera immunisation and disinfection, was introduced in Assam, and a noticeable reduction in the mortality rate was observed (reported by Pandit, 1951). However it was not clear which of these measures was responsible for the success. In 1934, Morison showed that cholera phage had no prophylactic value and was of dubious therapeutic use. The uncertain efficacy of phage therapy was prolonged and it was not until 1971 that a group of workers published an exhaustive study showing that phages given orally or intramuscularly had no clinical effect on the course of cholera in patients receiving standard rehydration therapy (Marcvk et al., 1971). The failure of phage therapy in cholera was largely attributable to reliance on the natural multiplication of the phages within the vibrios in the patient's intestine and it was found that each multiplication cycle was longer than the average transit time through the intestine in an actively purging patient. There were also problems

associated with predicting the phage sensitivity of vibrio strains likely to be encountered in an epidemic.

However, the idea of phage therapy has not died and a recent report from Poland (Medical Laboratory Technology, 1972) advocated the use of phages for treating dysentery caused by Sh. sonnei and Sh. flexneri. Phages have also been used in the treatment of experimental infections. Dubos, Straus and Pierce (1943) found that mice injected with Shigella dysenteriae intracerebrally could be protected from death by intraperitoneal administration of a suitable phage preparation. More recently, Bartell, Orr and Garcia (1968) found that fatal infection could be averted if mice, injected intraperitoneally with Ps. aeruginosa, were given specific phages simultaneously or soon after receiving the challenge dose.

In contrast to phage therapy, pyocine therapy has received very little attention. In 1969, Bird and Grieble reported that a single dose of pyocine increased the survival of chick embryos infected with Ps. aeruginosa from 3% to 46%, but crude pyocine was toxic to 11% of embryos that received pyocine alone. The nature of the pyocine used is not clear. The pyocinogenic strain was not induced and thus production was low. The pyocine activity was sensitive to trypsin but was sedimentable by ultracentrifugation and the molecular weight was estimated to be greater than 200,000. Thus the preparation had properties of both high and low molecular weight types of pyocine.

Merrikin and Terry (1972) examined the effects of pyocine on experimental infections in mice. Again, the type of pyocine used is not clear, but induction of the pyocinogenic strain was used. It was found that mortality of mice infected intravenously with Ps. aeruginosa could be reduced in some instances, when pyocine was administered by the same route. However, these authors did not record the numbers of mice involved nor the degree of protection achieved, and thus their findings are difficult to evaluate.

Pyocines have some obvious disadvantages as potential therapeutic agents, in that they have narrow activity spectra and there is a danger of sensitivity associated with the administration of foreign protein. However, in view of the increase in Ps. aeruginosa infections and the resistance of the organism to many antibiotics and disinfectants, the examination of pyocines as therapeutic agents may prove useful.

The aim of this investigation was to examine the possible therapeutic applications of pyocines to Ps. aeruginosa infections. Since three different classes of pyocines have been identified, this study was designed to characterise a representative of each class and to compare the effects of these selected pyocines on infections caused by a single sensitive strain of Ps. aeruginosa.

MATERIALS

MATERIALS

The materials used in the course of this investigation are listed below:

CHEMICALS AND MEDIA

All commercial dehydrated media were made up according to the makers' instructions.

Acrinol (rivanol)

Supplied by Koch-Light Laboratories

Colnbrook, Bucks, England.

Azoalbumin

Supplied by Sigma, Norbiton Station Yard, Kingston-upon-Thames, Surrey, England.

Blood agar

Oxoid Columbia agar CM331 with 5% human blood

(Oxoid Ltd., Southwark Bridge Road, London, S.E.1., England)

Casein

Fat-free, vitamin-free. Supplied by B.D.H. Chemicals Ltd., Poole, Dorset, England.

Cetrimide agar

Proteose peptone no. 3 (Difco)	20 g
Oxoid no. 1 agar	10 g
Glycerol	10 g
Distilled water	1000 ml

pH 7.2 Autoclave at 121°C for 15 min.

To 100 ml amounts of the above medium add:

1 ml 15% solution of K_2HPO_4 (anhydrous)

1 ml 15% solution of $MgSO_4.7H_2O$

1.5 ml 2% solution of cetrimide (B.P.)

Chromatography materials

DEAE - Sephadex A-25 and Sepharose 4B supplied by Pharmacia Fine Chemicals AB, Box 604, S-751 25 Uppsala 1, Sweden.

Deoxyribonuclease

DNase 11 (ex bovine spleen), lyophilised, salt free.

Supplied by Koch-Light.

Deoxyribonuclease agar

Oxoid CM 321

Egg yolk emulsion (concentrated)

Oxoid SR 47

Freund

Complete adjuvant. Difco, Detroit, Michigan, U.S.A.

MacConkey agar

Oxoid CM 7B

Mitomycin-C (Kyowa)

Supplied in 2 mg amounts by Dales Pharmaceuticals Ltd., Steeton, Keighley, Yorkshire, England.

Nutrient agar

Oxoid Columbia agar CM 331

A9 agar plates contained 9 ml nutrient agar in plastic Petri dishes, 85 mm in diameter.

Nutrient broth

Oxoid nutrient broth no.2 CM 67

$NB_{20}S$

20% nutrient broth in saline

Nembutal

Sterile solution of pentobarbitone sodium. One ml contains 60 mg nembutal. Supplied by Abbott Laboratories Ltd., Agro-Vet division, Queenborough, Kent, England.

Polyethylene glycol

Molecular weight 6,000. Pure. Supplied by Koch-Light.

Saline

Sterile physiological saline (0.85%)

SGB

Glutamic acid sodium salt		20 g
Yeast extract		1 g
$Na_2HPO_4.12H_2O$		5.64 g
$MgSO_4.7H_2O$	1% solution	10.0 ml
KH_2PO_4	1% solution	25.0 ml
$Ca(NO_3)_2.4H_2O$	1% solution	1.54 ml
Distilled water	pH 7.3	960 ml

Sterile glucose solution (0.5 g per ml) added to broth after autoclaving to give a final concentration of 1%.

THM buffer

0.02M-Tris HCl buffer containing 0.02M-$MgCl_2.6H_2O$

Trypsin

Difco 1:250

Tryptone soya agar

Oxoid CM 131

Tryptone soya blood agar

Tryptone soya agar with 5% human (or horse) blood.

EQUIPMENT

Brass burning blocks

Cross-sectional area of each block was 19 x 19 mm and the weight of each block was 119 g.

The block was suspended by a length of string through a loop in the top of the brass.

Cellulose acetate electrophoresis strips

Oxoid. 16 x 2.5 cm. 120μ thick.

Dialysis tubing

Visking seamless cellulose tubing. Size 18/32

Digital display unit

Supplied by Scientifica and Cook Ltd., 37-39 Ramsey Road, Acton, London, England.

Droppers

Platinum-tipped glass droppers calibrated to deliver 0.02 ml per drop were supplied by Astell Hearson, 172, Brownhill Road, Catford, London, England.

Homogeniser

Vertical drive, variable speed.

M.S.E. Manor Royal, Crawley, Sussex, England.

Needles and syringes

Disposable plastic syringes and Yale microlance needles supplied by Becton-Dickinson U.K. Ltd., York House, Empire Way, Wembley, Middlesex, England.

Orbital incubator

Supplied by Gallenkamp, Technico House, Christopher Street, London, England.

Photographic plates

Special lantern contrasty. Ilford Ltd., 53/54 Berwick Street, London, England.

Rotary film evaporator

EU-050 Supplied by Gallenkamp.

Spectrophotometers

Unicam Instruments Ltd., York Street, Cambridge, England.

Spreaders

Spreaders were made from glass capillary tubes. Each tube was 10 cm long and 0.1 mm in diameter and was bent at right angles 0.2 cm from one end. Both ends of the tube were sealed. The spreaders were stored in metal containers and sterilised in a hot air oven at 160°C for 1 hour.

The shorter arm of the spreader was used to distribute the fluid over the entire surface of the agar plate.

BACTERIAL STRAINS

Pseudomonas aeruginosa strains P14 and 6A were kindly supplied by Dr. R. Jones, Birmingham Accident Hospital, Birmingham, 15.

Ps. aeruginosa strains H41 and H108 were kindly sent by Professor B. Holloway, Monash University Melbourne, Australia.

All the other strains of Ps. aeruginosa used in this investigation were obtained from the strain collection in the Epidemiology Laboratory, Bacteriology Department, University of Edinburgh.

ANIMALS

*CFE mice were bred in Bacteriology Animal House, Medical School, University of Edinburgh.

*CBA mice were either bred in the Animal House or obtained from the Centre for Laboratory Animals, The Bush, Milton Bridge, Penicuik, Scotland.

MRC Hr and LAC Hr hairless mice were obtained from The Bush.

Porton rabbits were supplied by The Bush.

* Details of breeds are given in M.R.C. Standardised Laboratory Animals Manual Series 2 (1971).

SECTION 1

SURVEY OF STRAINS OF *PSEUDOMONAS AERUGINOSA* FOR THE PRODUCTION OF PYOCINES INHIBITORY TO THE SELECTED INDICATOR STRAINS

SECTION 1

Survey of strains of Pseudomonas aeruginosa for the production of pyocines inhibitory to the selected indicator strains

The pyocines of Pseudomonas aeruginosa may be divided into two major groups, primarily on the basis of their molecular weight. Those of low molecular weight (c. 10^5), by virtue of their size, diffuse easily through agar and pass through certain membranes. They are non-sedimentable in the ultracentrifuge and have not been resolved in the electron microscope. The second group consists of particles with a molecular weight in the range 10^6 - 10^7, which do not diffuse easily through agar or membranes, and can be sedimented by ultracentrifugation. Electronmicroscopic preparations reveal two types of particle in this group: (a) A complex particle resembling the headless version of a T-even coliphage, composed of a contractile sheath surrounding an inner core. (b) A smaller, filamentous particle resembling certain filamentous bacteriophages. These two kinds of particulate pyocines are not easily distinguishable except by electronmicroscopy, although the filamentous types more often diffuse through membranes while the large, contractile type of pyocine is held back.

The low molecular weight pyocines are also distinguishable

from those of high molecular weight by their sensitivity to heat and to proteolytic enzymes.

A survey of strains of Ps. aeruginosa was carried out to select those with pyocine activity against the chosen indicator strains, and to classify, by the above criteria, the kinds of pyocines that were produced.

Two indicator strains were examined initially, Ps. aeruginosa P14 and 6A, belonging to pyocine types 16 and 1 respectively. These strains have been studied in detail in Birmingham and are known to be virulent to mice.

METHODS

(1) The diffusion of pyocines in agar and their sensitivity to trypsin

Strains

Ps. aeruginosa indicator strains P14 and 6A were tested for pyocine sensitivity.

Ninety-four strains of Ps. aeruginosa from the laboratory collection were tested for their ability to produce pyocines active against the indicator strains. These strains were designated "pyocine producer strains".

All strains were stored on nutrient agar in screw-capped bijoux bottles at room temperature (c. 22°C) and plated out on to nutrient agar before use.

Media

Tryptone soya agar (at pH 8.0) and tryptone soya agar containing 0.05% trypsin (final concentration) were dispensed in 16 ml volumes into plastic Petri dishes.

Apparatus

A "multiple stab inoculator" was made from a honeypot lid, by passing nine straight Ni-chrome wires (each 4.5 cm long) through holes in the lid and fixing them with araldite. Each wire was placed 2 cm from its nearest neighbour to give a grid of 3 x 3 wires.

A tube-holder was made from a block of expanded polystyrene

to hold nine 3 in. x ½ in. tubes in the same pattern as the wires on the inoculator (fig. 1).

Growth of pyocine producer strains

The pyocine producer strains of Ps. aeruginosa were grown in 4 ml volumes of nutrient broth (NB) in 3 in. x ½ in. tubes for 4 hours at 37°C. The tubes were placed in the polystyrene holder and the cultures used to charge the sterilised wires of the inoculator. Tryptone soya agar plates (with and without trypsin) were inoculated by stabbing the charged inoculator into the agar to the bottom of the plate. The wires were re-charged with the cultures before inoculating each plate. The plates were incubated overnight at 32°C and then sterilised by inverting each plate over a honeypot lid containing filter paper soaked in chloroform. The depth of the agar in each plate was such that a seal formed between the surface of the agar and the rim of the honeypot lid. Thus the chloroform did not attack the plastic Petri dish (fig. 2). The plates were exposed to chloroform vapour for 15 min. and then left slightly open for 1 hour to allow the chloroform to disperse.

Indicator strain overlay

Molten nutrient agar (0.6% agar solution) was held in a waterbath at 45°C and distributed into 3 in. x ½ in. tubes in 3 ml aliquots. A 0.5 ml volume of a 4 hour NB culture of the indicator strain (P14 or 6A) was added to each tube of agar and mixed by rolling the tube between the hands. An indicator-seeded agar was poured over each sterilised plate of the pyocine producer strains. When the agar had set, the plates were

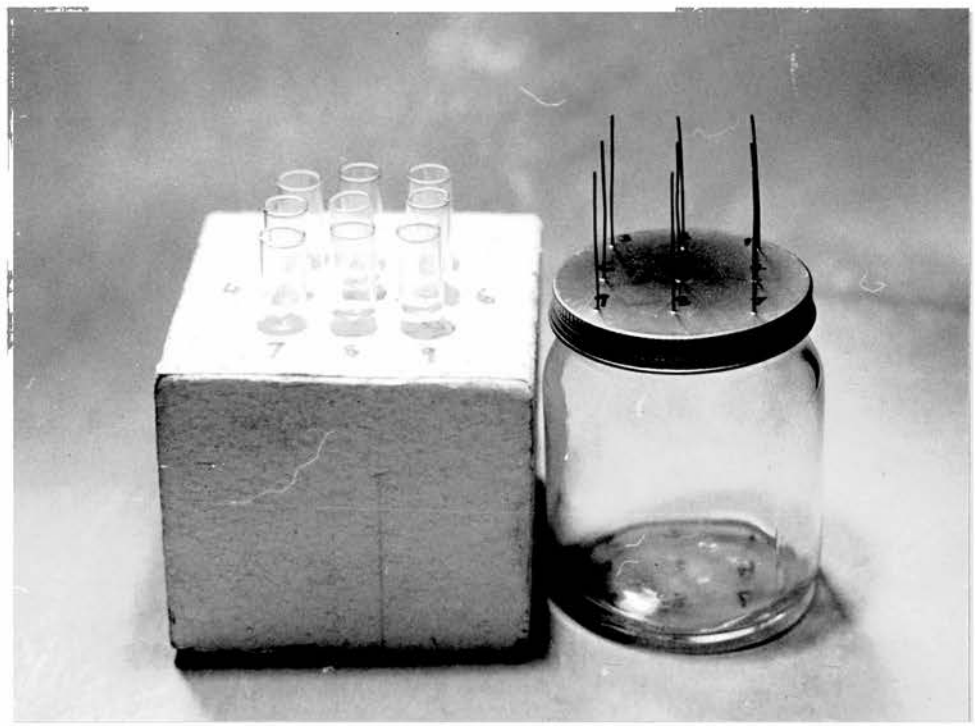


FIGURE 1 The multiple stab inoculator and tube-holder

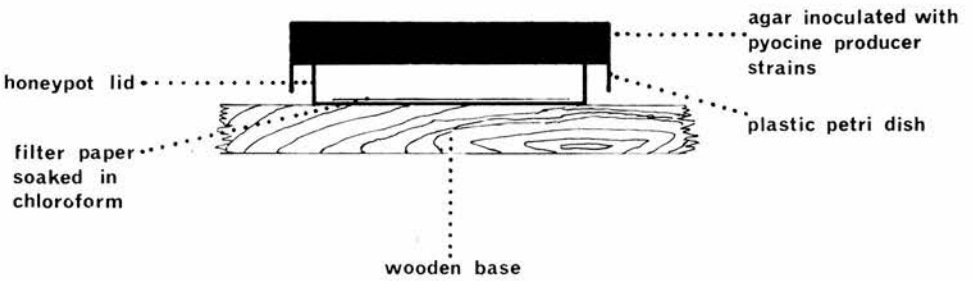


FIGURE 2 The sterilisation of bacterial growth on plastic Petri dishes

incubated at 37°C overnight.

The radius of the inhibition zone of the indicator strain was measured from the point of entry of the wire to the circumference of the zone. This measurement included the width of growth of the producer strain. The zone sizes in the presence and absence of trypsin were compared.

(a) The passage of pyocines through cellulose acetate membranes

Wahba (1963) suggested that some pyocines were able to pass through membrane filters while others were held back. Kohn (1966) introduced the use of cellulose acetate membranes to facilitate the removal of macroscopic growth in the pyocine typing procedure. However, this modification proved unsuitable for typing because some pyocines were incapable of passing through the membrane (Govan, 1968). Thus it was thought that cellulose acetate membranes might be useful for grouping pyocines according to their ability to pass through the membranes. This method has already been applied to a few pyocinogenic strains of Ps. aeruginosa (Govan, 1974b).

Cellulose acetate electrophoresis strips, cut into 8 cm lengths, were used. Each strip was laid diametrically across the surface of a tryptone soya blood agar plate, taking care that no air bubbles formed between the strip and the medium.

Pyocine producer strains that had inhibited indicator strains P14 or 6A in the previous experiment were used. Each strain was

carefully inoculated on to a cellulose acetate membrane using a bacteriological loop. Duplicate plates, without membranes, were also inoculated (fig. 3). All of the plates were incubated at 32°C overnight.

After incubation, the cellulose acetate strips were removed with sterile forceps and discarded. The bacterial growth on the plates without membranes was removed with a sterilised glass microscope slide. All the plates were sterilised by exposure to chloroform vapour for 15 min. and then left slightly open for 1 hour to remove any traces of chloroform.

Indicator strains P14 and 6A were grown in NB for 4 hours and a loopful of each culture was streaked across each plate at right angles to the original inoculum. The plates were incubated overnight at 37°C. The inhibition of the indicator strains was recorded.

Induction of pyocine producer strains

Pyocines are produced spontaneously by only a few cells in a population, but the number of cells producing pyocine can often be increased by inducing the pyocinogenic culture with mutagenic agents such as Mitomycin C or ultra-violet irradiation.

From the survey of strains described previously, 13 strains of Ps. aeruginosa that produced pyocines active against indicator strain P14 were examined for their inducibility. These strains were 19, 26, 41, 47, 62, 5837, 5866, 5873, 5882, 5893, H108, H41 and Ep672.

A 2 ml volume of an overnight NB culture of each pyocine

producer strain was used to inoculate 100 ml of SGB in a 2 l flask. The cultures were incubated in the dark in an orbital incubator at 32°C and 80 r.p.m. After 3 hours incubation, a solution of Mitomycin C was added to each culture to give a final concentration of 1.5 μg per ml, and incubation was continued for a further 5 hours; the flasks were then removed from the incubator and a 5 ml volume of chloroform was added to each culture and mixed well. After 15 min. the sterilised preparation was decanted and stored at 4°C. These preparations are referred to as stage I pyocine preparations.

Estimation of pyocine activity

A standard method of titrating pyocine preparations was used throughout this study. Lawns were prepared on A9 agar plates by flooding the surface of the plates with a 4 hour NB culture of strain P14, removing the excess culture with a pipette and allowing the lawns to dry. Doubling dilutions of the pyocine preparation were made in saline in a WHO plate. A 0.02 ml drop of each dilution was spotted on to the lawn, using a calibrated platinum-tipped dropper. When the drops had dried, the plates were incubated overnight at 37°C. The titre of pyocine activity, expressed in units per ml, was read as 50 times the reciprocal of the highest dilution causing complete inhibition of the indicator strain.

Stage I pyocine preparations of the 13 strains of Ps. aeruginosa were titrated in this manner and their activity against indicator strain P14 was recorded.

The effect of trypsin on stage I pyocine preparations

Pyocines were prepared to stage I by the method described previously, centrifuged at 1850g for 30 min. and the supernates were decanted. A 1% stock solution of trypsin was diluted in distilled water to give a solution containing 4 mg/ml. 0.5 ml aliquots of each pyocine preparation were mixed with 0.5 ml trypsin solution or 0.5 ml saline and incubated at 37°C for 1 hour. Equal volumes of trypsin and saline were also mixed and incubated. After incubation, each pyocine mixture was titrated on a lawn of indicator strain P14 and the plates were incubated overnight at 37°C. The activity of the pyocine preparations in the presence and absence of trypsin was recorded. The activity of the trypsin was confirmed by spotting the trypsin and saline mixture on to the gelatin emulsion of a photographic plate. This plate was incubated in a moist chamber at 37°C for 1 hour and then held at 4°C for a few minutes before swabbing with cold water. Clearing of the emulsion indicated the presence of trypsin activity.

The effect of heat on stage I pyocine preparations

The supernates of the stage I pyocine preparations, obtained in the previous experiment, were used. A 1 ml volume of each pyocine preparation, in a 3 in. x ½ in. tube, was gently agitated in a waterbath at 60°C for 10 min. After heating, the activity of the pyocine preparations was compared with that of unheated controls by titrating both test and control preparations on lawns of indicator strain P14.

Ultracentrifugation of stage I pyocine preparations

Pyocine was prepared to stage I as described previously and the preparations were centrifuged at 1850g for 30 min. The supernates were decanted into cellulose nitrate ultracentrifuge tubes and centrifuged at 100,000g for 2½ hours under refrigeration, using a Spinco angle 40 rotor. After ultracentrifugation, the supernates were removed and each deposit was resuspended in 1 ml of saline. The pyocine activities of the supernates and the resuspended deposits were estimated by titration on lawns of indicator strain P14.

Electronmicroscopy

Two methods were used for preparing the pyocines for viewing under the electron microscope. The negative staining technique of Brenner and Horne (1959) was employed.

(a) Pyocine particles alone

Stage I pyocine preparations were ultracentrifuged as described previously and the deposits were resuspended in 0.5 ml volumes of 1M - ammonium acetate (pH 7.0). Using a platinum loop, two loopfuls of this suspension were mixed with two loopfuls of a 2% potassium phosphotungstate solution (pH 7.0) on a clean glass microscope slide. A loopful of the mixture was transferred to the surface of a copper grid (NEW 200) and held for 30 s before the excess fluid was removed with blotting paper. The grids were allowed to dry in a dessicator.

(b) Pyocine particles adsorbed to cells of indicator strain P14

Stage I pyocine preparations were ultracentrifuged as described previously and the deposits were resuspended in 1 ml volumes of saline. 1 ml of resuspended pyocine was added to 3 ml of a 4-hour peptone water culture of strain P14 and incubated at 37°C for 20 min. The adsorption mixture was fixed by adding two drops of 20% formaldehyde and after 20 min. the mixture was centrifuged at 2600g for 45 min. The deposit was resuspended in 0.5 ml 1M - ammonium acetate (pH 7.0) and stained with phosphotungstate as described above.

RESULTS

Survey of strains of Pseudomonas aeruginosa for production of pyocines inhibiting the indicator strains P14 and 6A

(1) Diffusion of pyocines in agar and their sensitivity to trypsin

Ninety-four strains of Ps. aeruginosa were examined for their pyocine production against the indicator strains P14 and 6A by a stab inoculum technique. Thirty strains did not inhibit either of the indicator strains. Indicator strains P14 and 6A were inhibited by 56 and 22 of the producer strains respectively. The sizes of the inhibition zones in the presence and absence of trypsin are recorded in tables 1a and b.

TABLE 1a The influence of trypsin on the activity of 56 pyocine producer strains of Ps. aeruginosa against indicator strain P14

Strains	Radius of inhibition zone (mm)	
	without trypsin	with trypsin
Activity unaffected by trypsin		
30, 47, 57, 5821 5823, 5863, 5866, 5896, R485, PP430	2	2
12, 31, 45, 5837 5843, 5850	3	3
7	2-3	3
62	3	2-3
5820	2-3	2
5839	3-5	5
5849	3	3-4
5851	2-6	6
5873	1-2	2-3
5893	4-5	5
5902	3-5	4
5903	1-2	2
Activity enhanced by trypsin		
53, 5878, 5825, 5867 R21	1 2 1	2 3 3
Activity eliminated by trypsin		
5907	1	0
5882, 5883, 5908	2	0
54, 5884, 5893	3	0
H108, H41	4	0
13	3-4	0
41, 58	5	0
Ep672	6	0
26	7	0
Activity reduced by trypsin		
59	3	1
61	3	2
28, 5827, 5830	4	2
5875	4	3
19	5	2
5838	5	3
8, 5864	6	3

TABLE 1b The influence of trypsin on the activity of 22 pyocine producer strains of Ps. aeruginosa against indicator strain 6A

Strains	Radius of inhibition zone (mm)	
	without trypsin	with trypsin
Activity unaffected by trypsin		
12	2	2
13	3	3
30	2	2-3
61	2	2
5858	1-2	2
5887	2-3	3-4
R21	2	2
Activity enhanced by trypsin		
59	2	3
5849	2	3
5850	2	3
5855	1	2
R34	2	3
Activity eliminated by trypsin		
5843	2	0
5848	2	0
5853	1-2	0
5863	2	0
5879	4	0
5901	2	0
Activity reduced by trypsin		
47	3	2
50	3	2
5884	4	3
5908	3	2

(2) Diffusion through cellulose acetate membranes

The inhibition of the indicator strains P14 and 6A by the 44 pyocine producer strains grown on cellulose acetate membranes is shown in tables 2a and b, and an example is shown in fig. 3. Three strains, 5853, 5863 and 5903, did not inhibit either of the indicator strains in this experiment. Twenty-three strains produced pyocines that passed through the cellulose acetate membranes and inhibited indicator strain P14. Fifteen strains produced pyocines that were inhibitory to strain P14 but did not diffuse through the membranes. Similarly, 12 strains produced pyocines that passed through the membranes and inhibited indicator strain 6A, whereas the pyocines from eight strains were held back.

Although the inhibition was clear, some growth of the indicator strains was often observed in the inhibition zones and this is recorded in the tables.

From this survey of pyocine producer strains it was found that fewer strains of Ps. aeruginosa produced pyocines active against indicator strain 6A than against indicator strain P14. Therefore strain 6A was excluded from the subsequent investigations. Strain P14 has the additional advantage of belonging to an unusual pyocine type (16) (fig. 4) which will be easily distinguishable in later in-vivo studies.

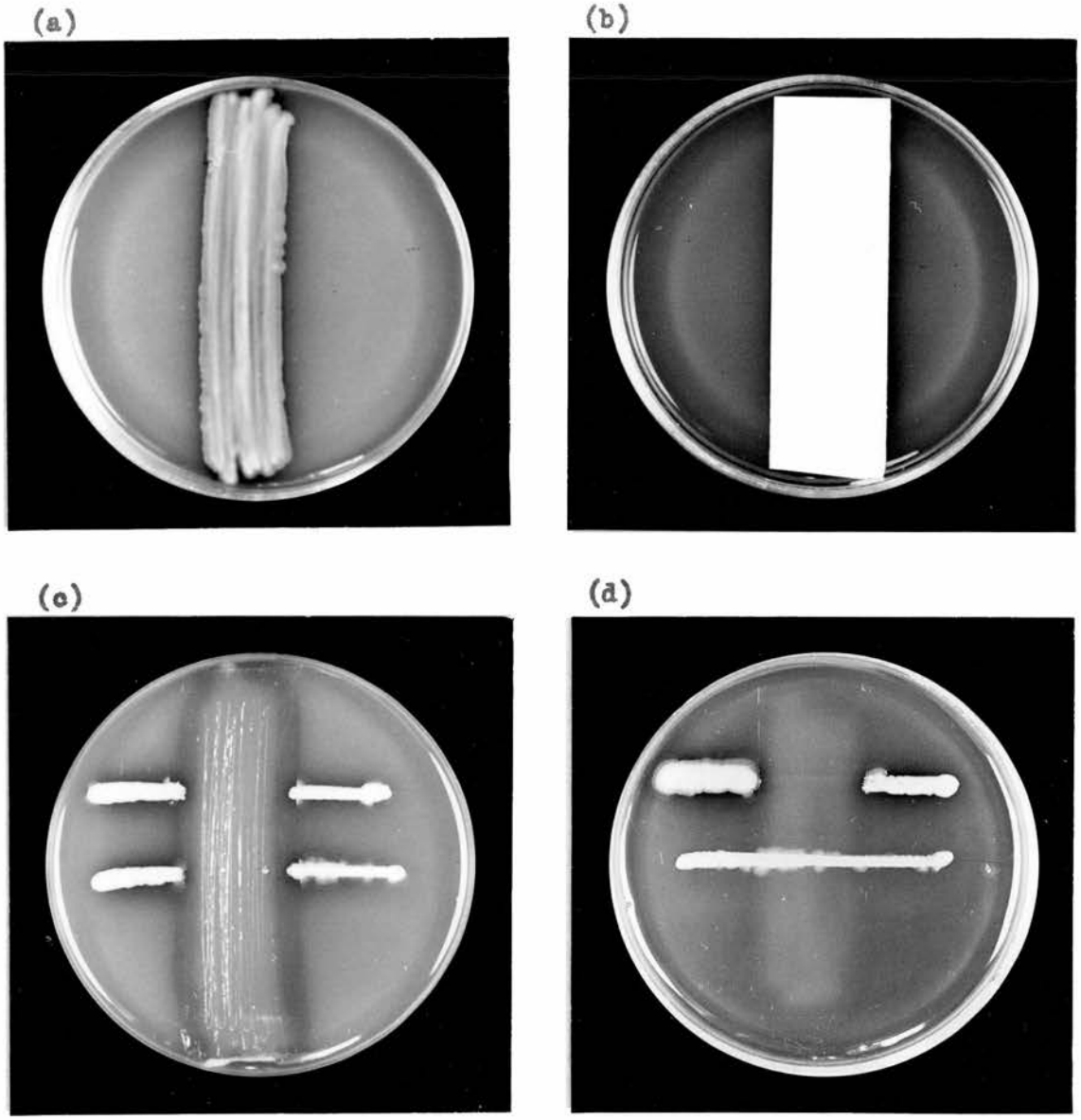


FIGURE 3 (a) Growth of pyocine producer strain on tryptone soya blood agar

(b) Growth of pyocine producer strain on a cellulose acetate membrane on tryptone soya blood agar

(c) Inhibition of indicator strains P14 and 6A by pyocines from the producer strain grown in the absence of a cellulose acetate membrane

(d) Inhibition of indicator strain P14 but not of strain 6A by pyocines from the producer strain grown on a cellulose acetate membrane

TABLE 2a Activity of 38 pyocine producer strains of Ps. aeruginosa against indicator strain P14. Producer strains inoculated on to the medium or on to a cellulose acetate membrane on the surface of the medium

Strains	Inhibition of indicator strain P14:	
	without membrane	with membrane
19, 26, 41, 58, 5821, 5873, 5882, 5887, H108, H41, Ep672	+	+
12, 13, 17, 18, 5843, 5848, 5898, 5908	+R	+R
5820, 5823, R21	+	+R
5866	+R	+
5837, PP430	+	-
30, 47, 59, 61, 5839, 5849, 5879, 5883, 5884, 5893, 5901, 5907, R485	+R	-

Key: + = inhibition of indicator strain P14

- = no inhibition of indicator strain P14

R = growth of resistant colonies of the indicator strain in the inhibition zone

TABLE 2b Activity of 20 pyocine producer strains of Ps. aeruginosa against indicator strain 6A. Producer strains inoculated on to the medium or on to a cellulose acetate membrane on the surface of the medium

Strains	Inhibition of indicator strain 6A:	
	without membrane	with membrane
17	+	+
12, 13, 5887	+R	+
18, 5823, 5843, 5882	+	+R
58, 5848, 5879, 5901	+R	+R
PP430	+	-
30, 47, 59, 61, 5849, 5858, 5908	+R	-

Key: + = inhibition of indicator strain 6A

- = no inhibition of indicator strain 6A

R = growth of resistant colonies of the indicator strain in the inhibition zone

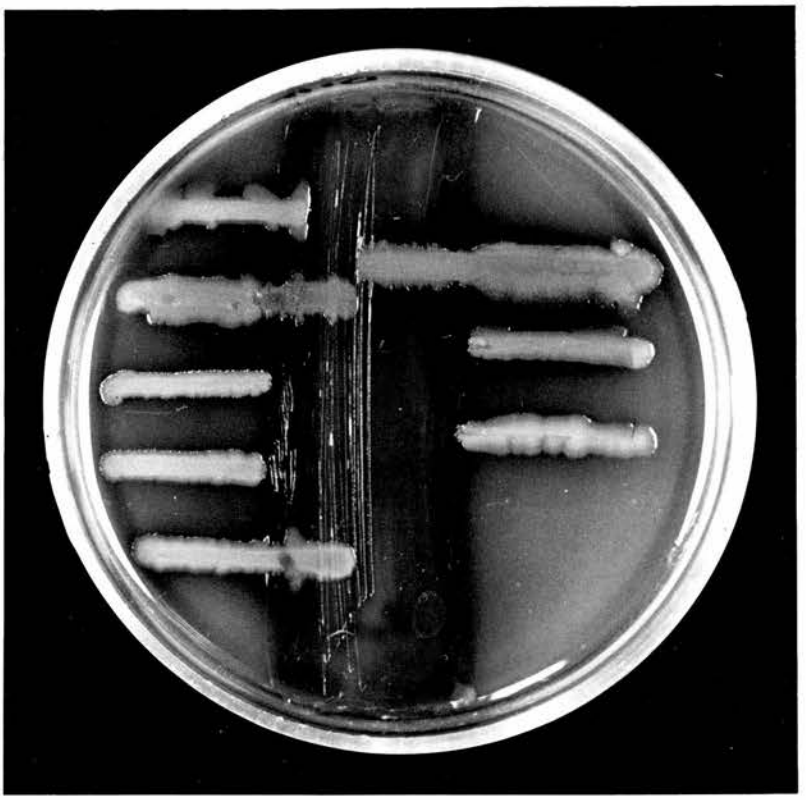


FIGURE 4 A typing plate showing the pyocine type 16 inhibition pattern produced by Pseudomonas aeruginosa strain P14

Induction of pyocine producer strains

Thirteen selected strains of Ps. aeruginosa were examined for their ability to produce pyocines after induction with Mitomycin C. The results are shown in table 3. Strains that gave pyocine titres greater than 1600 units per ml after induction were considered to be inducible. By this criterion, nine strains were inducible and only one of them (Ep672) also produced bacteriophage against indicator strain P14 as indicated by plaques in the indicator lawn. Strain Ep672 together with the non-inducible strains (26, 41, 5866, 5873) were not investigated further.

TABLE 3 Induction of 13 selected pyocine producer strains with Mitomycin C

Strain	Pyocine activity against strain P14 (units per ml)	
	without induction	with induction
19	< 50	3200
26	< 50	400
41	400	1600 *
47	1600	12,800-25,000
62	100	12,800
5837	100	6400
5866	< 50	100
5873	< 50	100-200
5882	800	50,000
5893	800	12,800
H108	< 50	3200
H41	< 50	3200
Ep672	< 50	3200 *

Key: * = bacteriophage active against strain P14 was also produced

The effect of trypsin on stage I pyocine preparations

The sensitivity of the induced pyocines to trypsin was examined. The treatment of stage I pyocine preparations with trypsin completely removed the inhibitory activity of the pyocines from strains 19, H41 and H108. The remaining pyocine preparations showed little change in activity. The results are given in table 4.

TABLE 4 The effect of trypsin on stage I pyocine preparations

Pyocine from strain	Activity of pyocine against strain P14 (units per ml)	
	untreated	after trypsin treatment
19	3200	0
47	50,000	25,000
62	3200	6400-12,800
5837	1600	1600
5882	50,000	50,000
5893	6400-12,800	3200
H108	3200-6400	0
H41	6400	0

Pyocine producer strains 19 and 5837 were not investigated further because they produced low titres of activity.

The effect of heat on stage I pyocine preparations

The effect of mild heat treatment on the activity of the selected stage I pyocine preparations is shown in table 5. The activity of pyocine preparations from strains 5882, H108 and H41 was markedly reduced by heating the preparations at 60°C for 10 min. The activity of pyocines from strains 47, 62 and 5893 was unchanged by the heat treatment.

TABLE 5 The effect of heat on the inhibitory activity of stage I pyocine preparations

Pyocine from strain	Activity of pyocine against indicator strain P14 (units per ml)	
	before heating	after heating (60°C, 10 min.)
47	3200-6400	3200-6400
62	6400	3200-6400
5882	50,000	800
5893	6400-12,800	6400
H108	1600-3200	100
H41	6400	100

Ultracentrifugation of stage I pyocine preparations

The selected stage I pyocine preparations were ultracentrifuged and the pyocine titres of the supernates and the resuspended deposits were assayed against indicator strain P14. The results of the titrations, given in table 6, show that the pyocines from strains 47, 62, 5882 and 5893 were sedimentable whereas those from strains H108 and H41 remained dispersed throughout the fluid after ultracentrifugation.

TABLE 6 Inhibitory activity of 6 pyocine preparations against strain P14 after ultracentrifugation

Pyocine from strain	Activity (units per ml) against strain P14 of:	
	supernate	resuspended deposit
47	100-200	50,000
62	0	25,000-50,000
5882	0	50,000,000
5893	1600	25,000-50,000
H108	800-1600	1600
H41	1600	800

Electronmicroscopy

Electronmicrographs of pyocine preparations from strains H108 and H41 did not show any pyocine particles. In an electronmicrograph of the pyocine from strain 5882 adsorbed to P14 cells, many filamentous particles were seen attached to the cell surface (fig. 5). Pyocine preparations from strain 47 showed a few contractile pyocines and some filamentous particles attached to a cell of P14. Strain 5893 appeared to produce contractile pyocines that attached to cells of strain P14.

FIGURE 5 An electronmicrograph showing the filamentous particles of pyocine 5882 attached to a cell of indicator strain P14 (x 100,000)

DISCUSSION

Pyocine production is recognisable when a suitable indicator strain is used to detect the inhibitory activity. There are two major groups of pyocines; the low molecular weight (small) pyocines and the high molecular weight group. The latter may be divided into contractile and filamentous types and these pyocines may be observed by electronmicroscopy. This is the only definite means of confirming their presence. The existence of the small pyocines is not so easily verified because they cannot be resolved in the electron microscope. If a pyocinogenic strain produces only a small pyocine then the pyocine is conspicuous by its absence in electronmicrographs and may be classified as "small". But a single strain of Ps. aeruginosa may produce more than one type of pyocine simultaneously and these may or may not attack the same indicator strain. Thus a contractile particle may be visible in the electron microscope while the inhibition of the indicator strain is due to a small (and therefore invisible) pyocine. In this instance, it may be necessary to remove the high molecular weight pyocine and examine the remaining material for inhibitory activity. However, such visual identification is not practicable for screening large numbers of pyocine producer strains and other properties of pyocines must be used to obtain a preliminary classification. The small pyocines are distinguishable from the high molecular weight group by their diffusibility and their sensitivity to heat and proteolytic enzymes.

In this survey, 94 strains of Ps. aeruginosa were examined for their ability to produce pyocines inhibitory to the selected indicator strains. The types of pyocine responsible for the inhibition were classified initially according to their diffusibility and their sensitivity to trypsin. From a point inoculum of a producer strain in agar, the pyocines diffused outwards and the extent of their diffusion was shown by overlaying the plate with a sensitive indicator strain. Strains that gave rise to wide inhibition zones, which were completely removed by trypsin, were tentatively classed as producing small pyocines. Conversely, producer strains that gave smaller inhibition zones which were unchanged by trypsin, were thought to be producing high molecular weight pyocines. It was not possible to distinguish between zones produced by contractile and filamentous pyocines. Some producer strains gave rise to inhibition zones in the indicator overlay that were reduced in size but not removed by trypsin. Such strains were thought to be producing a mixture of small and high molecular weight pyocines inhibitory to the indicator strain. A small number of producer strains gave rise to inhibition zones that were slightly enlarged in the presence of trypsin; a finding still to be elucidated.

From the results with indicator strain P14, the average radius of the inhibition zone produced by the postulated small pyocines was 3.6 mm, whereas the average for the high molecular

weight pyocines was 2.6 mm. The average radius of inhibition zones that were diminished in the presence of trypsin changed from 4.6 mm to 2.5 mm when the enzyme was incorporated into the medium. This supports the view that such zones are the result of a mixture of types of pyocines and the larger average radius (4.6 mm compared with 3.6 mm for small pyocines alone) could be an additive effect of more than one pyocine.

In the second part of the survey, the ability of pyocines to diffuse through cellulose acetate membranes was examined. Contractile pyocines have been shown to be retained by such membranes (Govan, 1968) while small and filamentous pyocines are able to diffuse through. Forty-four of the original 94 strains of Ps. aeruginosa were examined, and the results, together with those from the first part of the survey were used to postulate what types of pyocines were responsible for the inhibition of the indicator strains. Thirteen pyocinogenic strains were selected for further investigation : strains 47, 62, 5837 and 5893 gave small inhibition zones that were insensitive to trypsin and their pyocines did not diffuse through cellulose acetate membranes. These strains were thought to produce contractile pyocines. Strains 5866, 5873 and 5882 also produced small inhibition zones that were insensitive to trypsin (except the zone produced by 5882 which was trypsin sensitive) but the pyocines from these strains did diffuse through the membranes and therefore were thought to be of the filamentous type. Strains 19, 26, 41, H108, H41 and Ep672 were all suspected of producing small pyocines because they gave

wide diffusion zones which were sensitive to trypsin and they diffused through cellulose acetate membranes.

The eventual aim of this study was to examine the therapeutic uses of pyocines and it was felt that highly active pyocine preparations would be required. Although pyocines may be produced spontaneously by a few cells in a population, production is often increased by induction of the culture with a mutagenic agent such as Mitomycin C. The 13 pyocinogenic strains of Ps. aeruginosa selected from the preliminary survey, were examined for their inducibility and any production of bacteriophage which also attacked the indicator strain. Nine strains were found to be inducible but one of these also produced phage against indicator strain P14 and was therefore discarded.

Throughout this investigation, a critical dilution assay technique was used for estimating the inhibitory activity of pyocine preparations. This method, in which doubling dilutions of the pyocine preparation are spotted on to a lawn of the indicator strain, is technically easy to perform. However, accurate determination of the end-point, that is the highest dilution of the pyocine giving complete lysis of the indicator lawn, is sometimes difficult. For this reason, a single reading could not always be made and the results of titrations are occasionally given as ranging between two adjacent dilutions. More accurate methods for the determination of pyocine activity have been examined and these are usually based on measurement of

turbidometric changes that occur when a pyocine preparation is added to a sensitive cell suspension (Young and Stewart, 1971; Merrikin and Terry, 1973). Although providing more accurate results, this type of assay is more complex to perform.

Pyocines were prepared from the remaining eight producer strains, and the trypsin sensitivity of the extracted pyocine was examined. The results confirmed that pyocines from strains 19, H108 and H41 after induction were also trypsin-sensitive and thus probably of the small variety. The activity of the pyocines from the other strains (5882, 47, 62, 5837 and 5893) was not inhibited by trypsin at the concentration tested, therefore confirming the suspicion that these pyocines belonged to the high molecular weight group. Strains 19 and 5837 gave low titres of activity throughout and were not investigated further.

Ito, Kageyama and Egami (1970) found that heating at 60°C destroyed the activity of a small pyocine but a contractile pyocine was unaffected. Takeya et al. (1967) found that the activity of the filamentous pyocine 28 was almost completely destroyed by heating at 60°C for 10 min. The results of this experiment showed that pyocines from strains H108 and H41 were heat sensitive which supported the view that these were small pyocines. The pyocine from strain 5882 was thought to be of the filamentous type and its activity was also significantly reduced by heat treatment. Strains 47, 62 and 5893 were thought to produce contractile pyocines and this was supported by their

insensitivity to heat treatment.

The classification of the selected pyocine producer strains was endorsed by the results of ultracentrifugation and electronmicroscopy of the pyocine preparations. Strains H108 and H41 produced pyocines that were non-sedimentable and not visible in the electron microscope. These results, in conjunction with the diffusibility of these pyocines and their sensitivity to heat and trypsin, confirmed that the inhibition of indicator strain P14 by strains H108 and H41 was due to the production of small pyocines. Pyocines from strain 5882, that attacked strain P14, were clearly shown by electronmicroscopy to be of the filamentous type. Strain 47 was observed in the electron microscope to produce a mixture of contractile and filamentous pyocines whereas strain 5893 appeared to produce contractile particles only, which could be seen attached to cells of strain P14.

In summary, from the experiments in this section three pyocinogenic strains of Ps. aeruginosa were selected; each strain produced one type of pyocine particle inhibitory to the indicator strain. The strains chosen were : H108, which produced small pyocines; 5882, which produced filamentous pyocines, and 5893, which produced contractile pyocines. All these pyocines showed inhibitory activity against indicator strain P14.

SECTION 2

A STUDY OF THE PURIFICATION, CONCENTRATION AND TOXICITY OF SELECTED PYOCINES

SECTION 2

A study of the purification, concentration and toxicity of selected pyocines

Pseudomonas aeruginosa strains 5893, 5882 and H108 produce contractile, filamentous and small pyocines respectively which inhibit indicator strain P14. The induction of these three pyocinogenic strains and the method of estimation of their activity against strain P14 has been described, and in this section, methods for the concentration and purification of the pyocines were investigated and the toxicity of the preparations in mice was examined.

In the remainder of this study, pyocine 5893 refers to the pyocine produced by strain 5893 and inhibitory to strain P14; similarly for pyocine 5882 and pyocine H108.

METHODS

Removal of slime from stage I pyocine preparations

Ps. aeruginosa frequently produces large amounts of slime in broth culture and it was necessary to remove the slime in order to separate the cells from the pyocine-containing supernate. Three methods of slime removal were tested on 100 ml aliquots of stage I preparations of pyocines 5893, 5882 and H108.

Method I: Molar solutions of manganous chloride ($MnCl_2.4H_2O$) and sodium hydroxide were mixed in the ratio 2:1 and filtered through Whatman No. 1 filter paper. The filtrate was added to the pyocine

preparations to give final concentrations of either 2, 4, 6, 8 or 10%. The pyocine and filtrate were mixed vigorously and centrifuged at 1850g for 30 min.

Method 2: Deoxyribonuclease (DNase) was dissolved in distilled water to give a solution containing 100 µg/ml. One ml of this solution was added to a 100 ml volume of each pyocine preparation and incubated at 37°C for 1 hour. The activity of the DNase was confirmed by spotting the enzyme solution on to DNase agar and allowing the drops to dry. The plate was then flooded with 1M-HCl and clear zones appeared where drops of the active solution had been placed.

Method 3: A 2 ml volume of 1% aqueous acrinol was added to 100 ml volumes of the pyocine preparations.

After each treatment the activities of the pyocines were estimated by titration against strain P14.

Stage I pyocine preparations from which the slime had been removed were designated stage II.

Concentration and purification of stage II pyocine preparations

The ultimate aim of this study was to examine the therapeutic applications of pyocines in Ps. aeruginosa infections and therefore it was considered necessary to design a method of achieving high activity pyocine preparations. Since pyocines are thought to be of a protein nature, methods of concentration and purification used for proteins were tested. Two simple concentration

procedures were examined; the use of polyethylene glycol (PEG), and of a rotary film evaporator. Precipitation by ammonium sulphate and the use of ultracentrifugation as methods of achieving concentration and some degree of purification were also investigated.

(a) PEG

200 ml volumes of stage II pyocine preparations (slime removed by manganous chloride) were poured into sacks of dialysis tubing and the tubing was knotted and laid on a tray sprinkled with PEG. The sacks were completely covered with PEG and held at 4°C for 4 hours. After this time, the exuded fluid and excess PEG were discarded and the concentrated pyocine was removed from the dialysis tubing.

(b) Rotary film evaporation

100 ml volumes of stage II pyocine preparations were reduced to approximately 10 ml by rotary film evaporation at 37°C.

(c) Ammonium sulphate

50 g of ammonium sulphate were added to 100 ml volumes of stage II pyocine preparations (to give 75% saturation) and mixed well until all the salt had dissolved. The treated preparations were held at 4°C for 48 hours when the precipitate was collected by centrifugation at 1400g for 1 hour under refrigeration. The resulting supernates were discarded and the deposits resuspended in THM buffer. These suspensions were dialysed against the same

buffer at 4°C for at least 24 hours to remove any traces of ammonium sulphate.

(d) Ultracentrifugation

Stage II pyocine preparations were ultracentrifuged at 80,000g for 90 min. under refrigeration using a Spinco angle 40 rotor. The supernates were removed and the deposits were resuspended in 4 ml of THM buffer.

After each treatment, the activities of the pyocine preparations were estimated by titration against strain P14.

Storage of pyocines at different temperatures

The stability of the pyocine preparations at different stages in their purification was examined. Two ml volumes of pyocines 5893, 5882 and H108 at stage II and after PEG and ammonium sulphate treatment were stored in screw-capped bijoux bottles at room temperature (c. 22°C), in the refrigerator (c. 4°C) and in the deep-freeze (c. -20°C). The activity of the pyocines was checked initially and after 5 and 15 weeks storage.

Examination of the toxicity of pyocine preparations in mice

Stage II preparations of pyocines 5893, 5882 and H108 were tested, together with the concentrates of these pyocines prepared previously by PEG or ammonium sulphate treatment or ultracentrifugation. The activity and sterility of the preparations were confirmed prior to injection.

Pairs of mice were injected intravenously, intraperitoneally or subcutaneously with the pyocine preparations in their various forms. The mice received 0.5 ml volumes by the intraperitoneal or subcutaneous route and 0.3 ml volumes intravenously. The animals were weighed immediately before injection and at intervals thereafter until the 13th day of the experiment when they were killed by cervical dislocation.

The results of the toxicity testing of pyocine preparations in mice suggested that pyocine 5893 had some toxic properties. Therefore, preparations of this pyocine were examined in greater detail in the following four experiments to try to elucidate the toxic factor.

DETAILED STUDY OF PYOCINE 5893

(1) A comparison of the effects of induced and uninduced preparations of pyocine 5893 in mice

A culture of Ps. aeruginosa strain 5893 was grown in SGB and induced with Mitomycin C. A second culture was grown under the same conditions but without induction. Both cultures were purified to stage II and aliquots of the stage II preparations were treated with PEG or ammonium sulphate. The activity and sterility of the preparations were confirmed before use.

Twenty-eight male CBA mice were caged in groups of four and given food and water ad lib.. One group acted as controls and

these were injected subcutaneously with 0.5 ml of saline. Of the other six groups, three received induced preparations of pyocine 5893 at stage II, after PEG, or after ammonium sulphate treatment, and the other three groups were given the corresponding uninduced preparations. All injections were of 0.5 ml volume given by the subcutaneous route. The mice were weighed immediately before injection and at intervals thereafter until the 17th day of the experiment when the survivors were killed by cervical dislocation.

(2) Examination of induced and uninduced stage II preparations of pyocine 5893 for haemolysin and extracellular enzymes

Induced and uninduced stage II preparations of pyocine 5893 were tested for the presence of haemolysin, protease and lecithinase by the following methods.

(a) Haemolysin

Liu, Abe and Bates (1961) recommended human red blood cells (RBC) for the estimation of haemolysin activity, whereas Carney and Jones (1968) used horse RBC. In this study, both types of RBC were tested as indicators of haemolytic activity.

The RBC were washed three times in saline and 5% suspensions of the washed cells were prepared in saline. Doubling dilutions of induced and uninduced preparations of pyocine 5893 were prepared in duplicate in 0.2 ml volumes of THM buffer in WHO plates. Horse and human RBC were added to a set of dilutions in 0.2 ml

volumes and the plates were agitated well and incubated at 37°C for 2 hours. After incubation the haemolytic activity of the pyocine was read as the highest dilution causing complete lysis of the RBC.

(b) Protease

A 1% suspension of casein in THM buffer was allowed to stand in boiling water for 15 min. to dissolve the protein (Kunitz, 1947). Doubling dilutions of the induced and uninduced preparations of pyocine 5893 were prepared in 1 ml volumes in THM buffer, 1 ml of the cooled casein solution was added to each dilution, and the mixtures were incubated in a waterbath at 37°C for 5 min. After incubation 3 ml of 5% trichloracetic acid were added to each dilution and allowed to stand for 1 hour at room temperature. The samples were centrifuged at 1100g for 10 min. and the optical density of the resulting supernates was read at 280 nm in a Unicam SP500 spectrophotometer. A substrate control containing casein and saline and an enzyme control containing pyocine and saline were treated in the same manner as the test samples.

(c) Lecithinase

A 5% suspension of egg yolk (Oxoid) in saline was added in 1 ml aliquots to equal volumes of doubling dilutions of induced and uninduced preparations of pyocine 5893. After mixing well, the samples were incubated at 37°C overnight. The presence of lecithinase activity was detected by a clearing of the cloudy

suspension and a curdled surface layer after incubation.

(3) Fractionation of induced and uninduced pyocine 5893 preparations by gel filtration

A Pharmacia chromatographic column (dimensions 2.5 x 45 cm) was filled with a suspension of Sepharose 4B (Pharmacia) and washed for several hours with THM buffer at a pressure head of 34 cm.

A stage II preparation of pyocine 5893 was treated with PEG to reduce the volume from 100 ml to 5 ml. The concentrated pyocine was applied to the top of the column and allowed to run into the gel. The sample was then eluted with THM buffer and the eluate was collected at 10 min. intervals for 6 hours. The optical density of the samples at 280 nm was estimated using a Unicam SP500 spectrophotometer and pyocine activity was estimated by titration of each fraction against strain P14.

The Sepharose column was washed with buffer for 18 hours (c. 1000 ml buffer) and the fractionation procedure was repeated using an uninduced preparation of pyocine 5893, concentrated with PEG as described above. The optical density of the eluate was recorded automatically but the pyocine activity was not estimated.

(4) Fractionation of induced pyocine 5893 and assay of eluate samples for pyocine, protease and elastase activity

The gel filtration of an induced preparation of pyocine 5893 was repeated by the method described in experiment 3. The optical

density of the eluate at 280 nm was recorded automatically and samples were collected at 20 min. intervals using an automatic fraction collector. Each eluate fraction was assayed for pyocine, protease and elastase activity. Pyocine activity was estimated by titration of each fraction against indicator strain P14.

The protease assay was based on the method of Tomarelli et al. (1949). A solution of azoalbumin (Sigma) containing 25 mg/ml was made up in THM buffer and 0.5 ml aliquots were added to 0.5 ml of each eluate fraction and the reaction was allowed to proceed for 30 min. in a waterbath at 37°C. A 4 ml volume of 5% trichloracetic acid was added to each reaction mixture and the samples were centrifuged at 1100g for 20 min. A 2.5 ml volume of each supernate was mixed with an equal volume of 0.5M-NaOH and the optical density of the resulting suspension was read at 440 nm (Filter 2) in a Unicam SP1300 colorimeter.

The elastase assay was based on the method of Naughton and Sanger (1961). A suspension of elastin-Congo Red was prepared in THM buffer to contain 5 mg of substrate in 3.5 ml of buffer. One ml of each eluate fraction was added to 3.5 ml of the elastin-Congo Red suspension and incubated for 1 hour in a waterbath at 37°C. The reaction mixtures were centrifuged at 1100g for 20 min. and the optical density of the supernates was read at 495 nm (Filter 3) in a Unicam SP1300 colorimeter.

The eluate fractions containing the two peaks of pyocine activity were ultracentrifuged at 80,000g for 90 min. under

refrigeration using a Spinco angle 40 rotor. The supernates were decanted and the deposits were resuspended in 3 ml of THM buffer and both components were titrated against strain P14. The resuspended deposit of peak I and the supernate of peak II were examined for toxicity by animal inoculation. The eluate fractions containing the maximum elastase and maximum protease activity were also tested *in vivo*. The effects of these selected fractions were compared with those of unfractionated pyocine 5893.

Twenty-four female CFE mice were caged in groups of four and given food and water *ad lib*.. The mice were injected intraperitoneally with 0.5 ml volumes of fractions of a pyocine 5893 preparation. Group 2 received unfractionated pyocine 5893; group 3, the maximum protease fraction; group 4, the maximum elastase fraction; group 5, the pyocine peak I; and group 6, the pyocine peak II. The mice in group 1 acted as controls and were injected with 0.5 ml of THM buffer. The animals were weighed immediately before injection and daily thereafter until the 11th day of the experiment, when they were killed by cervical dislocation.

Extended survey of pyocine producer strains

Preparations of pyocine 5893 were found to have undesirable effects *in vivo* and it was difficult to obtain high titres of pyocine activity even after concentration. Therefore, it was decided to extend the survey of pyocine producer strains outlined in Section 1 in an effort to find another strain of *Ps. aeruginosa*

that produced contractile pyocines that inhibited indicator strain P14. Seventy-seven strains of Ps. aeruginosa were tested by the methods described in Section 1 and those strains that were suspected of producing contractile pyocines were examined further. Strain 1577 was selected and the properties of its pyocine preparation were compared with those of pyocine 5893.

Toxicity testing of pyocine 1577

A 400 ml volume of pyocine 1577 was prepared to stage II and 200 ml of this preparation were reduced to 4 ml by treatment with PEG. Fifty g of ammonium sulphate were added to a further 100 ml of the stage II pyocine, mixed well, and allowed to stand at 4°C for 24 hours. The precipitate was collected by centrifugation at 1400g for 1 hour under refrigeration and resuspended in 6 ml of THM buffer. The resuspended material was dialysed against the same buffer at 4°C overnight.

A 96 ml volume of stage II pyocine 1577 was ultracentrifuged at 80,000g for 90 min. under refrigeration, using a Spinco angle 40 rotor. The supernates were discarded and the deposits were resuspended in a total volume of 6 ml of THM buffer.

The toxicities of the stage II pyocine 1577 and the concentrates prepared by PEG or ammonium sulphate treatment or ultracentrifugation were tested in mice. The activity and sterility of each preparation was confirmed before use.

Male CFE mice were caged in groups of four and injected intraperitoneally (0.5 ml) or subcutaneously (0.3 ml) with one of the pyocine preparations. Control mice received THM buffer. After subcutaneous injection, the mice were massaged at the site of injection. The mice were weighed immediately before injection and regularly thereafter until the 10th day of the experiment, when they were killed by cervical dislocation and examined at necropsy for any obvious pathological changes.

Further purification of pyocine 1577

The results of the toxicity testing of preparations of pyocine 1577 were not entirely satisfactory and although the original plan was to design a method of pyocine preparation that only required a few days to complete, in view of the toxicity problems, further purification steps were examined. The method is set out below:

Stages in the preparation of purified pyocine

Day 1 Three 200 ml volumes of SGB culture of Ps. aeruginosa strain 1577 were induced with Mitomycin C by the standard method. After incubation, the cultures were stored at 4°C overnight (stage I).

Day 2 The pyocine-containing supernate was treated with a filtrate of $MnCl_2$ + NaOH to give a final concentration of 4%. After mixing well, the slime precipitate was deposited by centrifugation at 1600g for 50 min. and the supernate was decanted (stage II). 250 g of

ammonium sulphate were dissolved in 500 ml of pyocine-containing supernate and held at 4°C for 24 hours.

Day 3 — The ammonium sulphate-treated pyocine was centrifuged at 2100g for 60 min. at 4°C. The supernate was discarded and the deposit was resuspended in 25 ml of THM buffer. The resuspended material was dialysed against two changes of the same buffer in 72 hours at 4°C (stage III).

Day 6 — The dialysed pyocine was centrifuged at 1500g at 4°C and the supernate was decanted and ultracentrifuged at 80,000g for 90 min. under refrigeration, using a Spinco angle 40 rotor. The resulting supernate was discarded and the deposits were resuspended in a total volume of 12 ml of THM buffer (stage IV).

Day 7 — Ion-exchange chromatography of stage IV pyocine: 10 g of DEAE-Sephadex A-25 were swollen in 250 ml of THM buffer for 48 hours at room temperature and used to pack a chromatographic column (Pharmacia; 30 x 1.5 cm). The packed column was washed for several hours with THM buffer and allowed to stand overnight.

A 6 ml sample of a stage IV preparation of pyocine 1577 was run into the top of the ion-exchange column and the system was closed for 1 hour to allow the pyocine to adsorb to the resin. The column was then washed with

THM buffer for 5 hours (*e.* 200 ml buffer). The pyocine was eluted with a sodium chloride gradient (0 - 1.0M-NaCl in THM buffer) and the eluate fractions were collected at 15 min. intervals by an automatic fraction collector. Elution was allowed to proceed overnight, during which time 68 fractions were collected with an average fraction volume of 6.5 ml.

Day 8 The protein content of the eluate samples from the ion-exchange column was estimated by reading the optical density (O.D.) of the samples at 280 nm in a Unicam SP 8000 spectrophotometer. The eluate samples with high O.D. readings were titrated against strain P14 and the remainder of the samples were spotted on to lawns of P14.

Day 9 The eluate samples that were found to contain high activities of pyocine were dialysed against THM buffer for 5 hours (stage V) and then ultracentrifuged at 80,000*g* for 90 min. under refrigeration, using a Spinco angle 40 rotor. The deposits was resuspended in THM buffer (stage VI), and the activity of the pyocine was estimated by titration against strain P14.

Toxicity testing of pyocine 1577 at various stages of purification

Twenty-four male CBA mice were caged in groups of four and given food and water *ad lib.*. Each group received pyocine 1577

from one of the stages of the purification procedure (stages II-VI) and the mice in the remaining group were injected with THM buffer. The activity and sterility of the preparations was confirmed before use. All the injections were of 0.5 ml volume, given intraperitoneally. The mice were weighed immediately before injection and daily thereafter for 13 days.

RESULTS

The removal of slime from stage I pyocine preparations

Three methods for removing slime from pyocine preparations were examined and the inhibitory activities of the pyocines after the different treatments are shown in table 7. Deoxyribonuclease and acrinol did not markedly reduce the activity of the pyocines but neither did they remove the slime from the preparations. Therefore, they were rejected as slime removal agents. The $MnCl_2$ + NaOH filtrate removed the slime from pyocine preparations efficiently but at higher concentrations of the filtrate, the pyocine activity was diminished, probably due to co-precipitation of the pyocines with the slime. Therefore a final concentration of filtrate of 4% was adopted as a standard. After centrifugation to remove the slime precipitate, some $MnCl_2$ remained in the pyocine-containing supernate, but this was removed by dialysis against distilled water. After slime removal, the pyocine preparations were designated stage II preparations.

TABLE 7 Removal of slime from stage I pyocine preparations

slime removal agent	activity of pyocine against strain P14 (units per ml)		
	5893	5882	H108
$MnCl_2$ +NaOH filtrate			
at 2%	12,800	200,000	3200
" 4%	6400-12,800	100,000	3200-6400
" 6%	1600-3200	100,000	3200
" 8%	800-1600	50,000	1600
" 10%	200	50,000	1600
DNase	3200	200,000	6400
Acrinol	800-1600	200,000	12,800
Untreated control	6400	200,000	6400

Concentration and purification of stage II pyocine preparations

Polyethylene glycol (PEG) and rotary film evaporation were examined as methods for concentrating stage II pyocine preparations. Precipitation with ammonium sulphate and the use of ultracentrifugation for concentrating the pyocines and achieving some degree of purification was also investigated. The activities of the pyocines after the different treatments are shown in table 8. Rotary film evaporation was found to be unsuitable for concentrating pyocine preparations because the pyocine activity was considerably reduced or completely removed after the treatment.

TABLE 8 The activities of pyocines 5893, 5882 and H108 at different stages of concentration and purification

stage of pyocine preparation	activity of pyocine against strain P14 (units per ml)		
	5893	5882	H108
stage I	12,800	50,000	6400
stage II	12,800	50,000	3200-6400
after PEG	200,000	1,600,000	6400
after ammonium sulphate	100,000	1,600,000	6400
after ultra-centrifugation	12,800†	400,000†	1600*

Key: † activity of resuspended deposit
* activity of supernate

Storage of pyocine preparations at different temperatures

Samples of pyocine preparations at various stages of concentration and purification were stored at different temperatures

and their activities examined 5 and 15 weeks later. The results are shown in table 9.

In general, the purified preparations showed a greater loss of activity than the stage II preparations and all the samples lost their activity during storage at room temperature. Preparations of pyocine H108 were the least stable and only retained their activity after 15 weeks if stored in a deep-freeze (-20°C). However the high molecular weight pyocines retained their activity better at 4°C.

TABLE 9 The activities of pyocines 5893, 5882 and H108 against strain P14 after storage at 22°C, 4°C and -20°C for 5 and 15 weeks

pyocine preparation	Duration of storage (weeks)	Activity of pyocine against strain P14 (units per ml) at temperature of storage (°C)		
		22	4	-20
5893 stage II	0	12,800	12,800	12,800
	5	50,000	50,000	50,000
	15	12,800	25,000	50,000
5893 after PEG	0	200,000	200,000	200,000
	5	6400	400	6400
	15	200	800	1600
5893 after ammonium sulphate	0	100,000	100,000	100,000
	5	6400	50,000	0
	15	0	25,000	0
5882 stage II	0	200,000	200,000	200,000
	5	50,000	25,000	200,000
	15	12,000	100,000	50,000
5882 after PEG	0	1,600,000	1,600,000	1,600,000
	5	2,000,000	2,000,000	2,000,000
	15	100,000	400,000	1,600,000
5882 after ammonium sulphate	0	1,600,000	1,600,000	1,600,000
	5	100,000	800,000	50,000
	15	3200	1,600,000	0
H108 stage II	0	1600	1600	1600
	5	0	800	1600
	15	0	100	800
H108 after PEG	0	6400	6400	6400
	5	0	100	6400
	15	0	0	100-200
H108 after ammonium sulphate	0	6400	6400	6400
	5	0	400	6400-12,800
	15	0	0	800

Toxicity testing of preparations of pyocines 5893, 5882 and H108 in mice

The results of injecting various types of pyocine preparation intraperitoneally, intravenously and subcutaneously into mice are shown in table 10. The activities of the relevant pyocine preparations are those shown in table 8.

One of the two mice injected intraperitoneally with pyocine 5893, concentrated by PEG, died. One mouse of each pair injected intraperitoneally with PEG and ammonium sulphate-treated pyocine H108 died. Pyocine 5882 caused no visible adverse effects.

Lesions developed around the neck and forelegs of some of the mice injected subcutaneously with pyocine 5893 (fig. 6). One mouse that received pyocine H108 suffered similarly but had recovered by the end of the experiment. Mice that developed lesions were not killed at the end of the experiment (13th day) but left alive so that the progress of the lesions could be observed. On the 10th day, the lesions were swabbed, but cultures yielded no bacterial growth and after about 20 days the lesions were healing naturally.

Lesions were not observed in mice injected with pyocine 5882 or THM buffer.

TABLE 10 Toxicity testing of preparations of pyocines 5893, 5882 and H108 by various routes of injection in pairs of mice

Pyocine	Stage of pyocine preparation	Route of injection	Number of deaths per pair of mice	Number of lesions per pair of mice
5893	stage I	SC	0	0
"	"	IP	0	0
"	after PEG	SC	0	2
"	"	IP	1	0
"	after ammonium sulphate	SC	0	2
"	"	IP	0	0
"	after ultra-centrifugation	SC	0	2
"	"	IP	0	0
"	"	IV	0	0
5882	all stages	all routes	0	0
H108	stage I	SC	0	0
"	"	IP	0	0
"	after PEG	SC	0	1
"	"	IP	1	0
"	after ammonium sulphate	SC	0	0
"	"	IP	1	0
"	after ultra-centrifugation	SC	0	0
"	"	IP	0	0
"	"	IV	0	0
Controls	THM buffer	SC	0	0
"	"	IP	0	0
"	"	IV	0	0

Key: SC = subcutaneous
IP = intraperitoneal
IV = intravenous

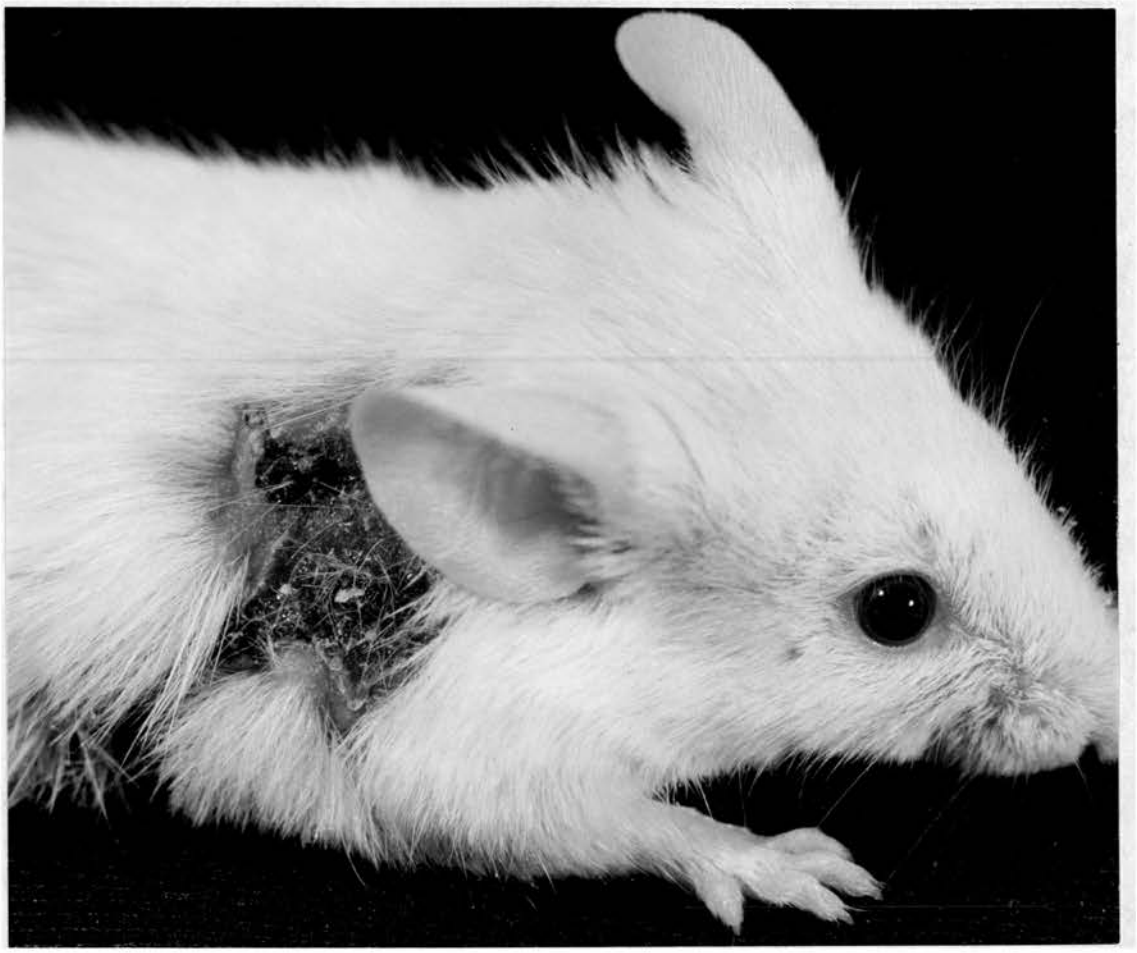


FIGURE 6 Lesion on a mouse ten days after subcutaneous injection of pyocine 5893

DETAILED STUDY OF PYOCINE 5893

(1) The effects of induced and uninduced preparations of pyocine 5893 in mice

The results of the previous experiment showed that six of the eight mice that received pyocine 5893 subcutaneously developed sores and one of the eight mice injected intraperitoneally died. In this experiment, the effects of induced preparations of pyocine 5893 were compared with those of uninduced preparations; the latter having low pyocine activity. The activities of the pyocine preparations administered to the mice are shown in table 11.

TABLE 11 The inhibitory activities of induced and uninduced preparations of pyocine 5893 administered to mice

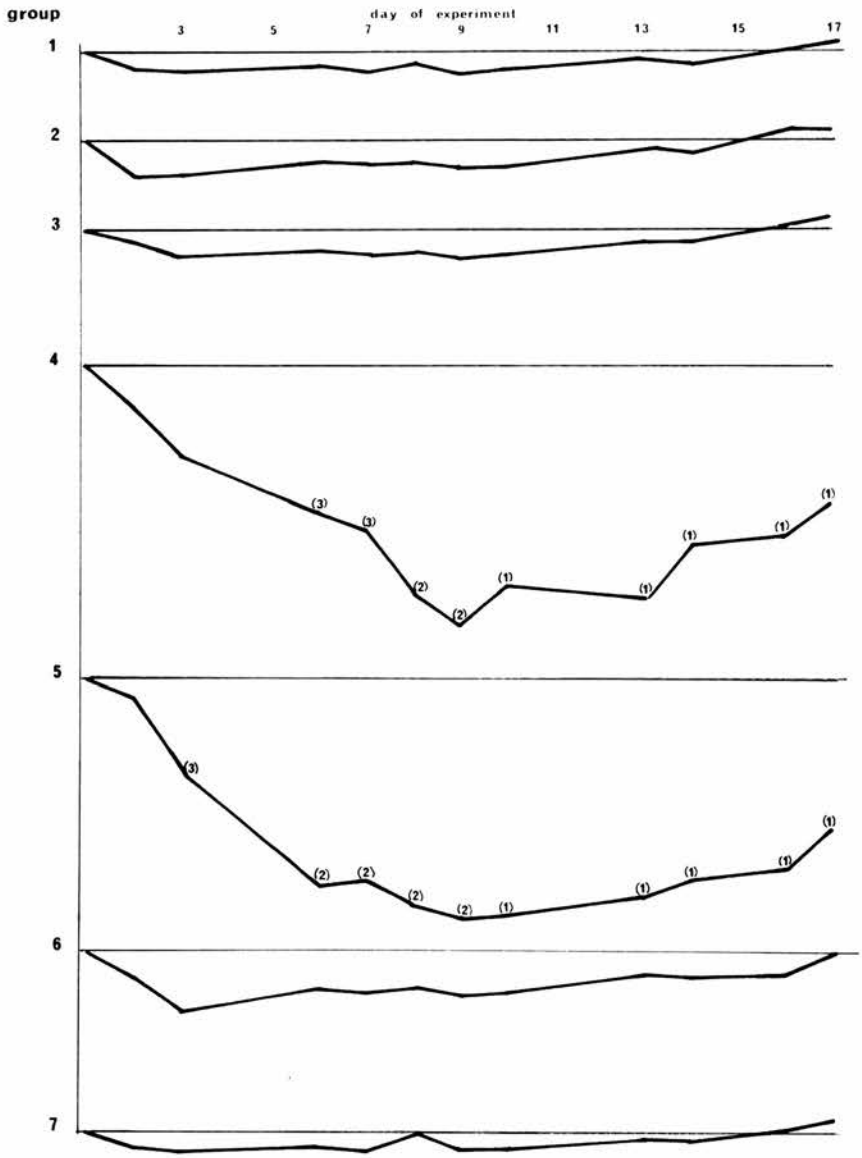
stage of pyocine preparation	activity of pyocine 5893 against strain P14 (units per ml)	
	induced	uninduced
stage II	6400 (1)	400 (4)
after PEG	25,000-50,000 (2)	1600 (5)
after ammonium sulphate	800 (3)	1600 (6)

Figures in brackets indicate mouse group number

The changes in average weights of the groups of mice and the time of death are shown in fig. 7. The average weight of a group relative to its initial weight was taken as a measure of the state of health of the group. There were no deaths among the mice that received induced preparations of pyocine 5893 but three of the four mice died in groups that were injected with uninduced pyocine 5893 at stage II or after PEG treatment. The mice were all injected subcutaneously but no surface lesions were observed.

FIGURE 7

Changes in average weights of groups of four mice injected with induced and uninduced preparations of pyocine 5893



baseline for each group is the initial average weight of the group

figures in brackets indicate the number of surviving mice where this is less than 4

(2) Extracellular enzyme and haemolytic activity of induced and uninduced stage II preparations of pyocine 5893

There was no evidence of any haemolytic or lecithinase activity in either the induced or uninduced preparations of pyocine 5893.

The protease assay showed a slight rise in optical density of the test samples above the controls, indicating that some of the casein substrate had been digested and thus some proteolytic activity was present in both induced and uninduced preparations of pyocine 5893.

(3) Fractionation of induced and uninduced preparations of pyocine 5893

Samples of induced and uninduced preparations of pyocine 5893 concentrated by PEG treatment, were fractionated on a Sepharose 4B gel filtration column and the optical density of the eluate was recorded. The pyocine activity of the eluate fractions of induced 5893 was also estimated and the results are shown in fig. 8.

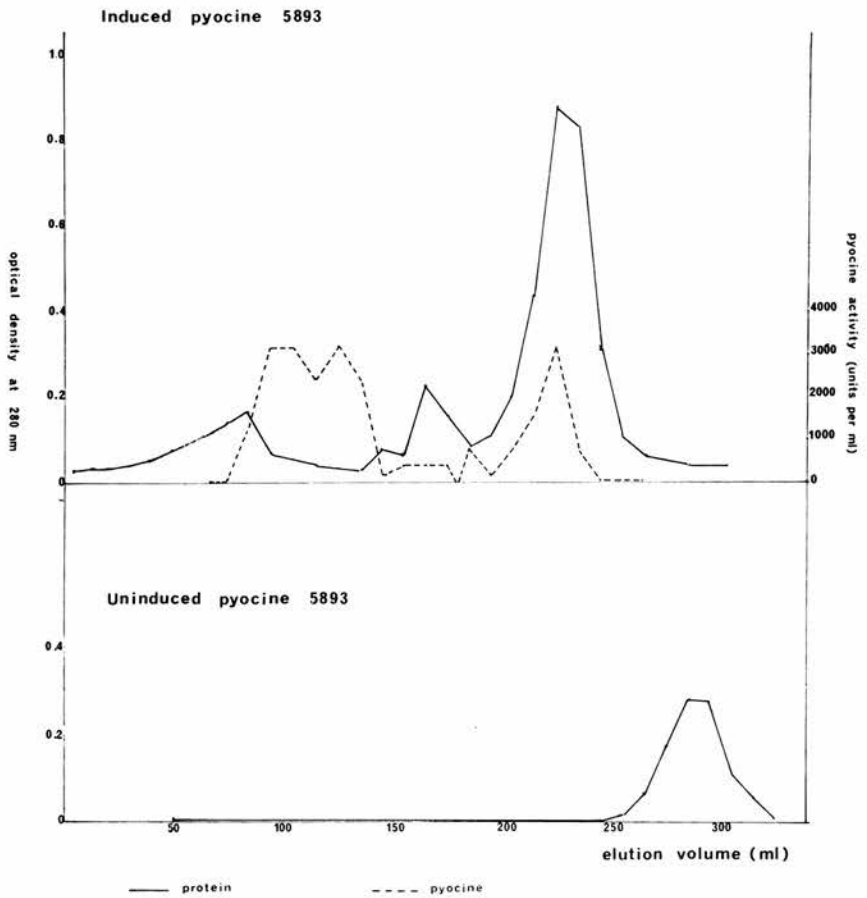
(4) Toxicity of fractions of an induced preparation of pyocine 5893 in mice

The fractionation of induced pyocine 5893 was repeated and the eluate samples were assayed for pyocine, protease and elastase activity. The results are given in table 12 and fig. 9.

The changes in average weights of the groups of mice injected with fractions of the pyocine 5893 preparation are shown in fig. 10. Group 5, which received pyocine peak I, were the only animals that were visibly unwell and this observation was borne out by their loss of weight. This group showed the greatest weight loss but the other groups of mice also lost weight initially. The samples containing high protease and high elastase activity

FIGURE 8

Fractionation of induced and uninduced preparations of pyocine 5893 on a Sepharose 4B gel filtration column



appeared to have less adverse effects than the pyocine-containing samples or the unfractionated pyocine 5893.

At necropsy, the organs of all the mice appeared normal except the spleens, which were often pulpy and sometimes enlarged.

TABLE 12 Assay of pyocine, protease and elastase activity of eluate samples from the fractionation of induced pyocine 5893 on Sepharose 4B

Eluate sample	pyocine activity (units per ml)	protease activity O.D. at 440 nm	elastase activity O.D. at 495 nm
1	0	0	0.025
2	0	0	0.02
3	0	0	0.03
4	0	0	0.015
5	0	0	0.015
6	100-200	0	0.025
7	6400-12,800	0.005	0
8	3200	0.005	0
9	1600	0.001	0.003
10	800	0.002	0
11	200-400	0.01	0
12	800	0	0
13	3200	0.005	0
14	6400	0.02	0
15	6400	0.005	0
16	400	0.015	0
17	0	0.002	0
Unfractionated 5893	200,000	0.022	0.175

FIGURE 9

Fractionation of a pyocine 5893 preparation and assay of eluate samples for protein, and protease, elastase and pyocine activity

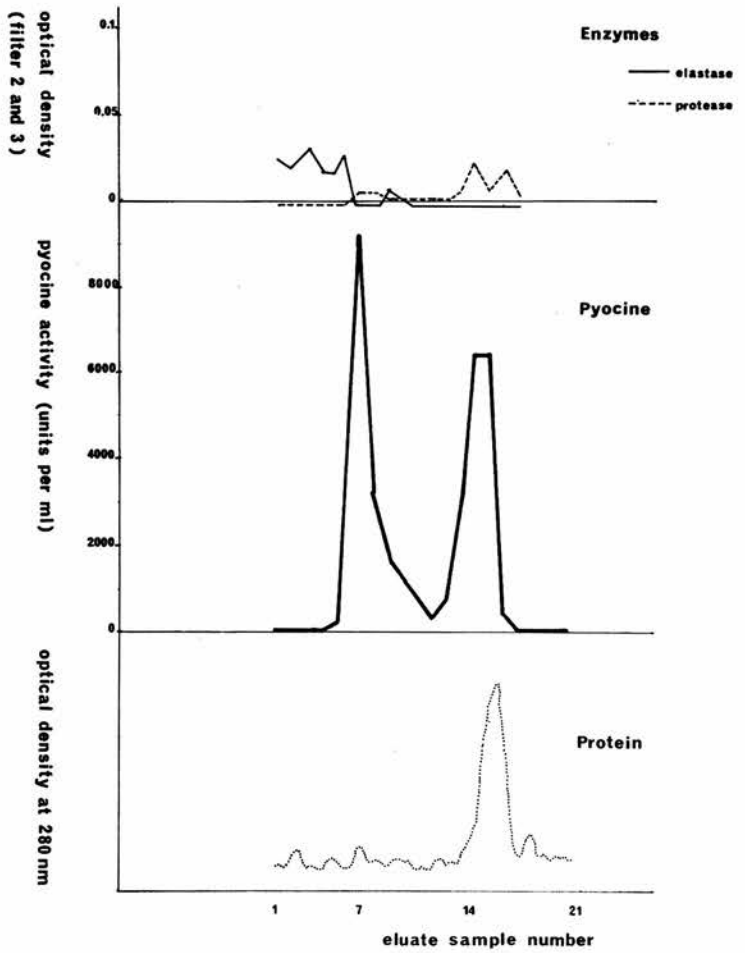
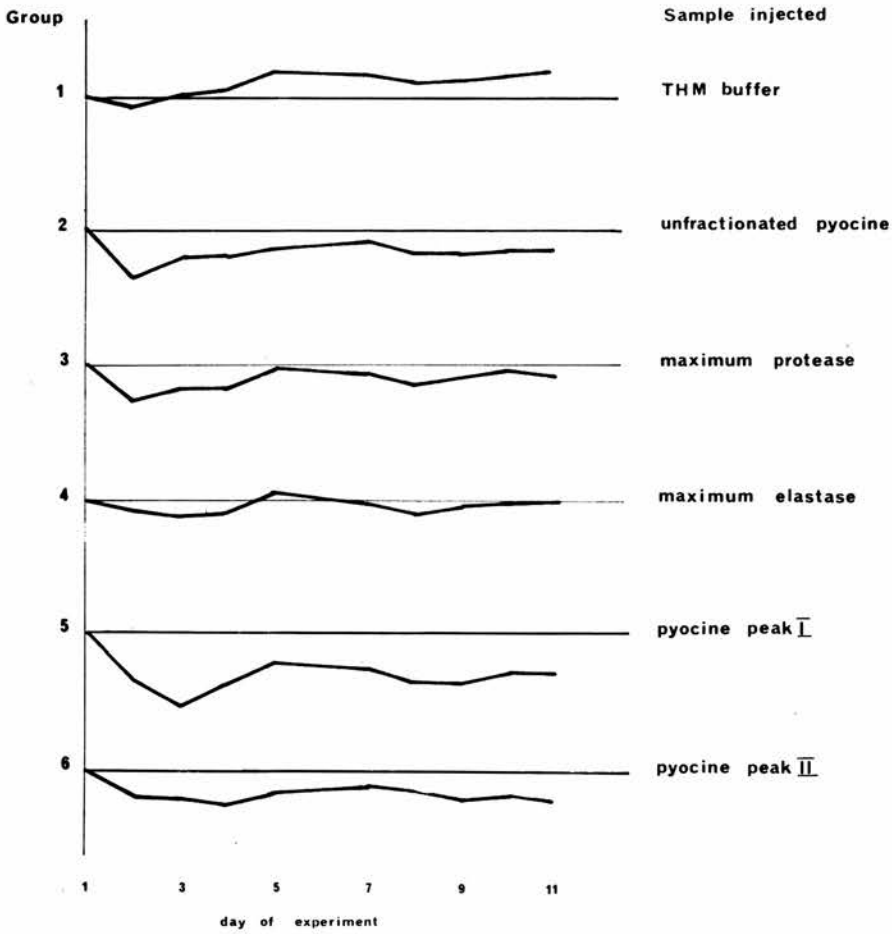


FIGURE 10

Changes in average weights of groups of four mice injected with fractions of an induced preparation of pyocine 5893



Baseline for each group is the initial average weight of the group

Extension of the initial survey for another strain producing contractile pyocines

Studies of pyocine 5893 in mice suggested that the toxic effects observed might have been due to the pyocine itself and not to other contaminating protein. Strain 5893 was therefore discarded as the representative contractile pyocine producer. From a further survey of pyocine producer strains, using the methods described in Section I, Ps. aeruginosa strain 1577 was selected. Pyocine 1577 was prepared to stage II and its properties were compared with those of pyocine 5893 and are tabulated below (table 13).

Electronmicrographs of pyocine 1577 showed clearly many contractile pyocine particles attached to cells of indicator strain P14 (fig. 11).

TABLE 13 A comparison of the properties of pyocines 1577 and 5893

Treatment	Activity of pyocine against strain P14 (units per ml)	
	1577	5893
Heat (60°C for 10 min.)	50,000	3200
Control (room temperature)	25,000-50,000	12,800
Trypsin (4 mg/ml; 60 min.) 37°C	25,000	3200
Control (37°C; 60 min.)	50,000-100,000	6400
Ultracentrifugation (80,000g; 90 min. deposits resuspended in 6 ml THM buffer)	deposit: 3,200,000	25,000-50,000
	supernate: 25,000	1600

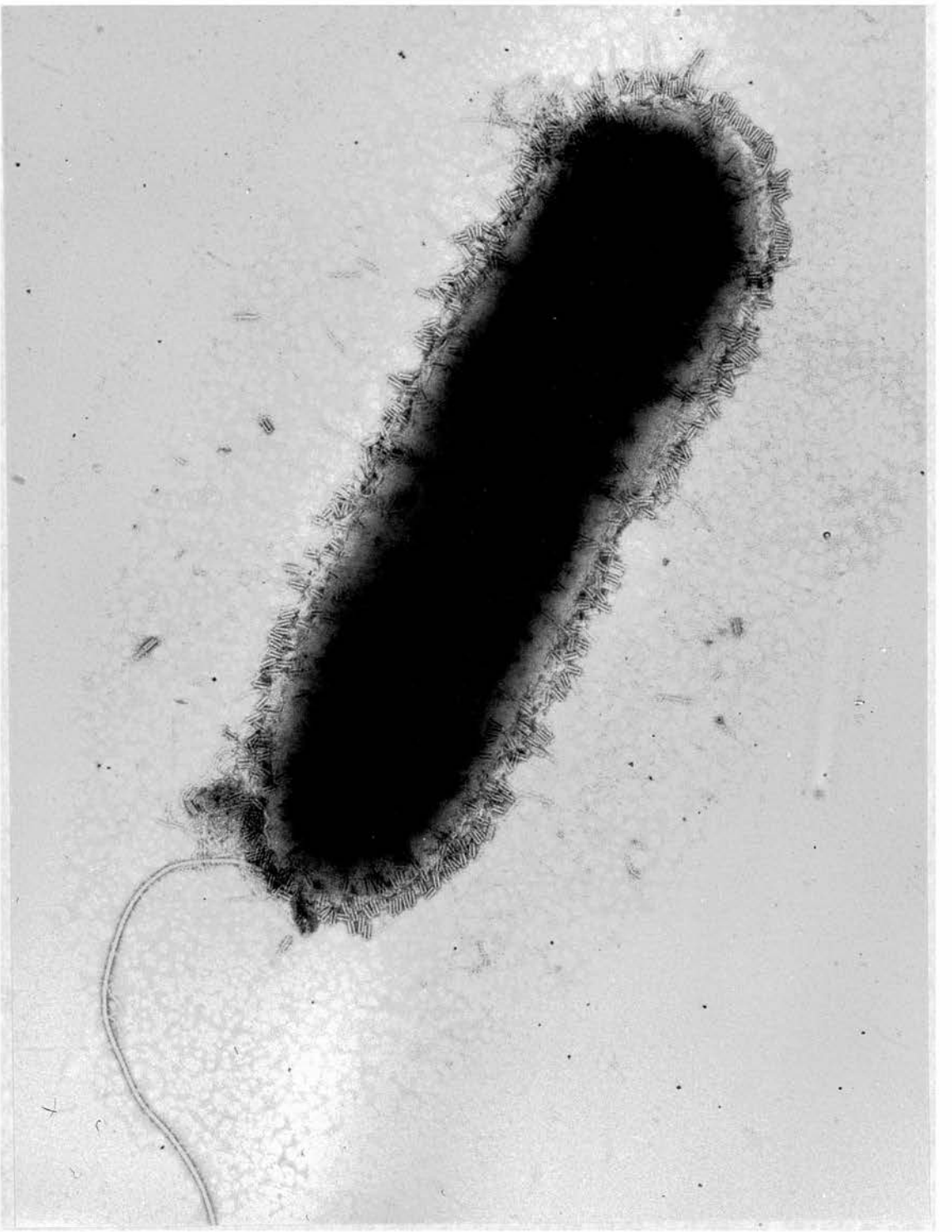


FIGURE 11 An electronmicrograph showing the contractile particles of pyocine 1577 attached to a cell of indicator strain P14 (x 50,000)

Toxicity testing of pyocine 1577

The inhibitory activities of the pyocine 1577 preparations administered to the mice and the average weight changes of the groups of four animals are shown in fig. 12.

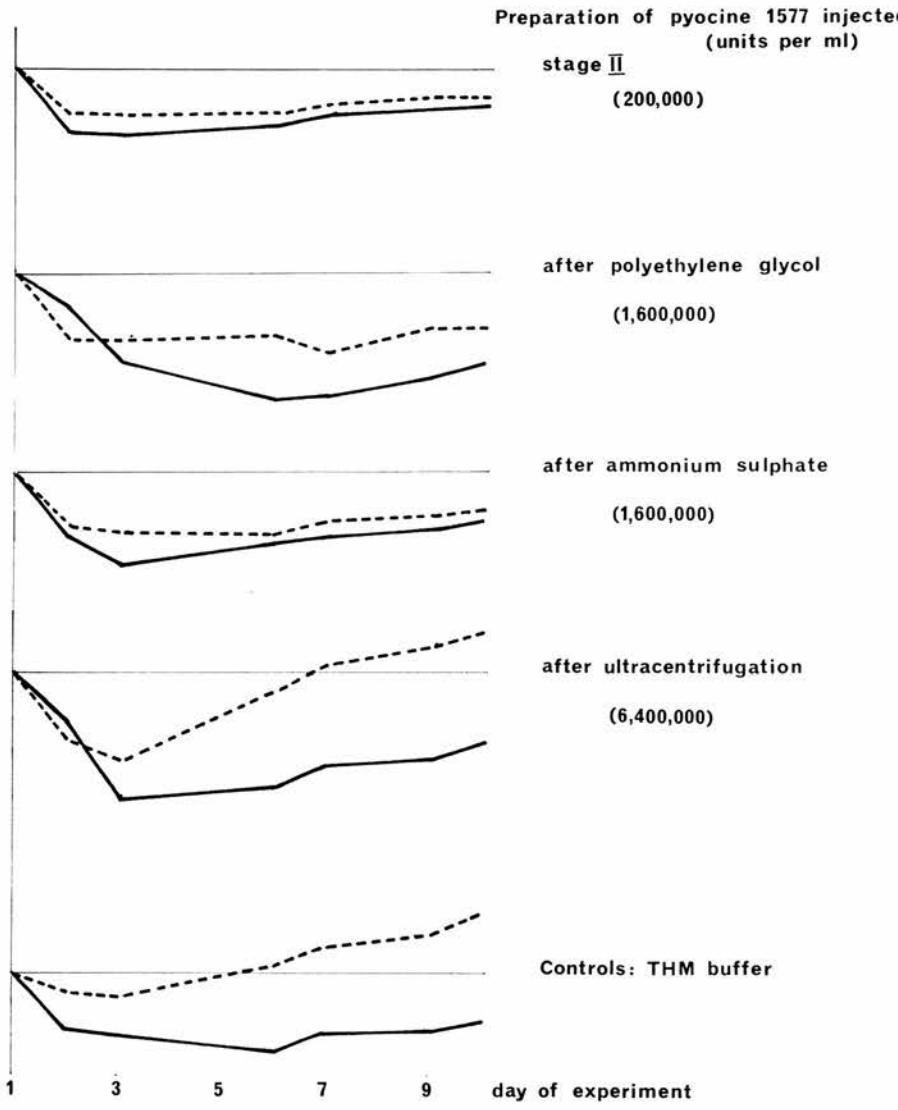
All the groups lost weight during the first two days after injection with pyocine. The control animals also showed a weight loss but this was smaller than that of the test groups. Mice injected intraperitoneally lost more weight than those that received pyocine subcutaneously.

Two mice injected intraperitoneally with PEG-concentrated pyocine were dead on the 6th day. One death also occurred in the group of mice inoculated with ammonium sulphate-treated pyocine, but at necropsy, the gut appeared distended and death may have been caused by injection into the gut. No deaths occurred in the groups of mice injected subcutaneously with pyocine preparations and no surface lesions were observed.

Post-mortem examination of the mice revealed no gross pathological changes.

FIGURE 12

Changes in average weights of groups of four mice injected with pyocine 1577 intraperitoneally or subcutaneously



—— mice injected intraperitoneally ---- mice injected subcutaneously

Baselines represent initial average weights of groups of mice

The purification of pyocine 1577 and toxicity testing at different stages of purity

The elution profile of a stage IV preparation of pyocine 1577 from a DEAE-cellulose ion-exchange column is shown in fig. 13. The maximum pyocine activity was eluted at a sodium chloride concentration of *c.* 0.17M.

Samples of pyocine 1577 from stages II-VI of the purification procedure were tested for toxicity to mice. The activities of the pyocine preparations injected are given in table 14. The numbers of surviving mice and the daily average weights of the groups are shown in table 15 and fig. 14.

TABLE 14 The activities of the preparations of pyocine 1577 administered to mice

group of mice	stage of pyocine preparation	activity of pyocine 1577 against strain P14 (units per ml)
1	II	200,000
2	III	400,000-800,000
3	IV	3,200,000
4	V	12,800
5	VI	6,400,000-12,800,000

Three of the four mice that received stage IV pyocine died, whereas only one mouse succumbed in the group that received stage VI pyocine, of higher activity. There were no deaths in any of the other groups, but all the mice showed some loss of weight. This may have been because some of the animals fought badly and the control mice eventually had to be caged individually.

FIGURE 13

Fractionation of pyocine 1577 on DEAE-cellulose:

optical density of eluate fractions and elution profile of pyocine

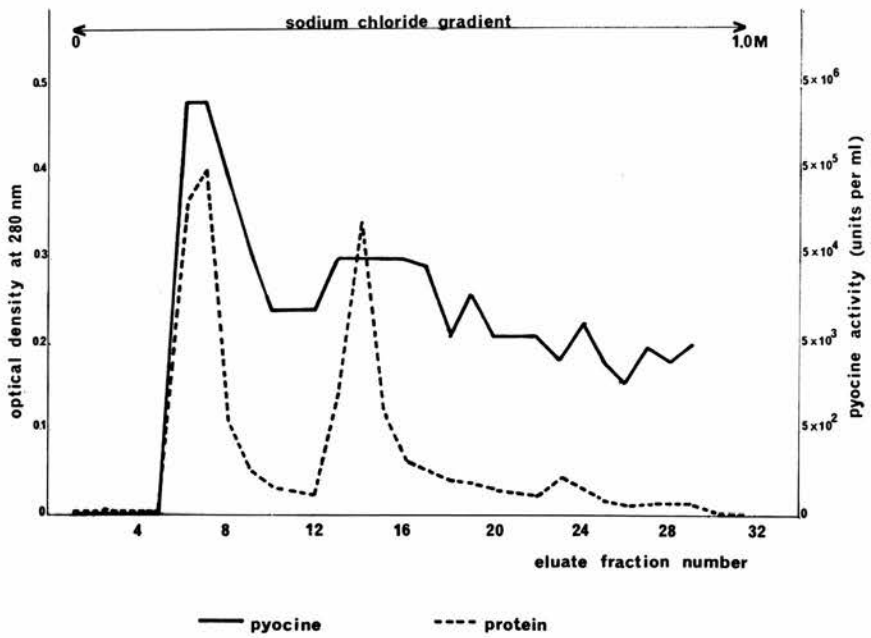
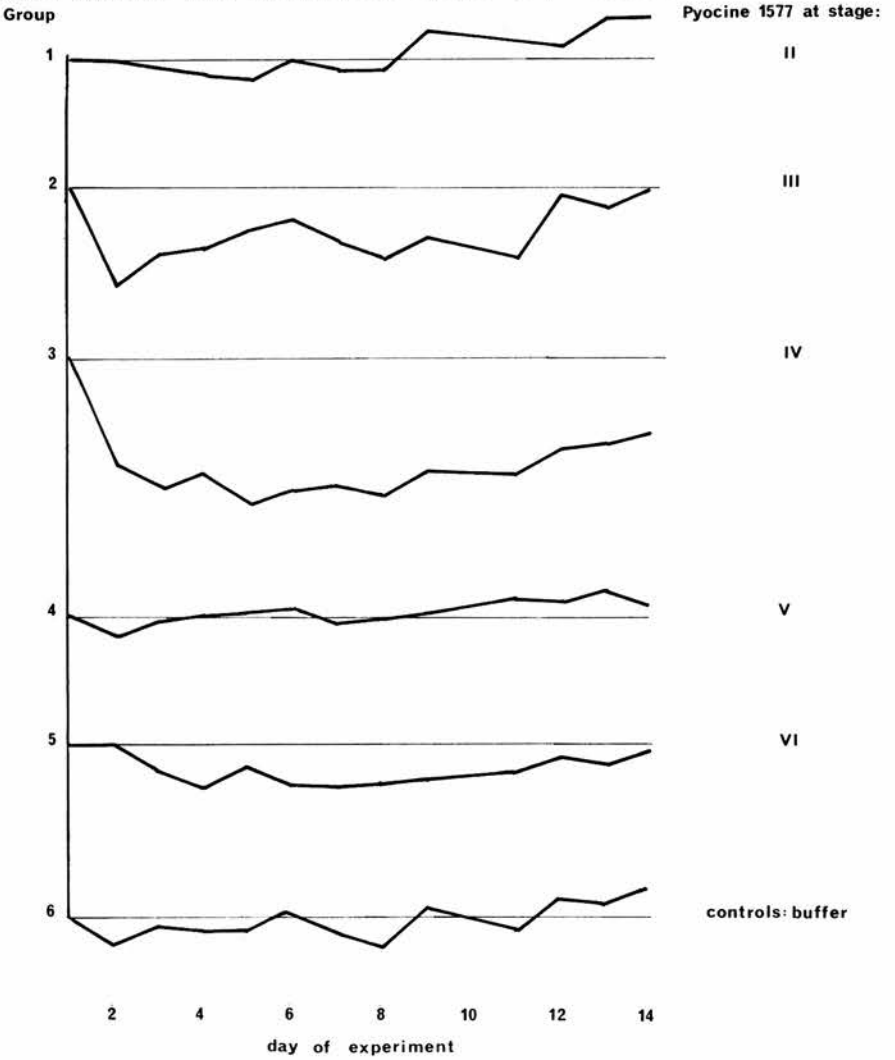


TABLE 15 The numbers of surviving mice and the average weights of the groups of mice injected with preparations of pyocine 1577

Group		Day number: 1	2	3	4	5	6	7	8	9	11	12	13	14
1	Number of surviving mice	4	4	4	4	4	4	4	4	4	4	4	4	4
	Average weight (g)	38.5	38.4	36.4	37.7	37.6	38.4	38.0	38.0	39.8	39.3	39.2	40.7	40.8
2		4	4	4	4	4	4	4	4	4	4	4	4	4
		40.1	35.6	37.2	37.3	38.2	38.7	37.6	37.0	37.8	36.9	39.8	39.4	40.1
3		4	3	2	2	1	1	1	1	1	1	1	1	1
		37.1	32.2	31.1	31.7	30.4	31.1	31.4	30.8	32.2	31.8	33.0	33.2	33.7
4		4	4	4	4	4	4	4	4	4	4	4	4	4
		40.2	39.3	40.1	40.3	40.4	40.6	39.9	40.1	40.5	41.1	41.0	41.5	40.8
5		4	4	4	4	3	3	3	3	3	3	3	3	3
		38.6	38.6	37.4	36.6	37.6	36.8	36.7	36.8	37.1	37.5	38.2	37.9	38.4
6		4	4	4	4	4	4	4	4	4	4	4	4	4
		37.1	35.9	36.7	36.5	36.6	37.3	36.4	35.9	37.6	36.7	38.0	37.9	38.5

FIGURE 14

Changes in average weights of groups of four mice injected with pyocine 1577 at different stages of purification



Baselines represent initial average weights of the groups

DISCUSSION

Early studies of bacteriocines were of a descriptive nature and concentrated mainly on the detection and assay of these inhibitory agents. More recently there has been an increasing interest in the molecular biology of bacteriocines and concurrently, descriptions of suitable purification procedures have been reported. The purification of a contractile pyocine preparation was described in detail by Kageyama and Egami (1962) and this method has become the basis for many of the published purification procedures. The purification of filamentous pyocines has not been studied in detail but is similar to the method used for contractile pyocines (Takeya et al., 1969). In a recent study of a small pyocine, Ohkawa, Kageyama and Egami (1973) advocated the method of Kageyama and Egami (1962) with certain modifications designed to overcome the instability of small pyocines. The main qualification was that all the procedures should be carried out at 0-4°C and that prolonged dialysis should be avoided because of the instability of the small pyocine at low salt concentrations.

Purification of a pyocine-containing supernate is necessary because Ps. aeruginosa elaborates a wide variety of extracellular growth products, including slime and enzymes. Although pyocines are active in unpurified preparations, it was felt necessary to obtain purified pyocines for studies of the inhibitory activity of these agents in vitro and in vivo. Furthermore, since it

has been shown that the bactericidal capacity of a pyocine preparation is proportional to the amount of pyocine present, (Kageyama, Ikeda and Egami, 1964) concentrated pyocine preparations were required. But purification and concentration procedures are often lengthy and since a fresh batch of pyocine would be required for each in-vivo experiment, it seemed desirable to keep the preparation time to a minimum. Thus a compromise was sought between the degree of purification and concentration of the pyocine preparations required and the length of time such procedures entailed.

The methods of purification examined in this study were based on those of Kageyama and Egami (1962) and various modifications reported by other authors were also tested.

The removal of slime is an important initial stage in the purification of pyocine preparations, not only because the slime contaminates the pyocine, but also because its presence hinders the separation of bacterial debris, and makes the handling of pyocine preparations difficult. The slime material has also been shown to be toxic to animals (Callahan, Beyerlein and Mull, 1964). Various methods for the removal of slime have been described. Kageyama and Egami (1962) found that treatment of a pyocine preparation with manganous chloride was effective in precipitating the viscous material, but it was necessary to add sodium hydroxide simultaneously to maintain the pH at about 7.4. The divalent manganese cation specifically precipitates nucleic

acids, but the pyocine does tend to be precipitated with the slime when manganous chloride is used. To avoid this loss of pyocine activity, Yui, Ishii and Egami (1969) recommended the addition of aqueous acrinol to the pyocine preparation. This method has the disadvantage that traces of acrinol remain in the treated preparation and can only be removed by passage through a carboxy-methyl (CM) cellulose ion-exchange resin. Since the slime of Ps. aeruginosa is composed largely of deoxyribonucleic acid (DNA), treatment of a pyocine preparation with DNase was found to be an effective method of slime removal (Ito, Kageyama and Egami, 1970).

In this laboratory, a modified method of manganous chloride treatment has been used successfully (Brown, 1973). Molar solutions of manganous chloride and sodium hydroxide are mixed in the ratio 2:1 and filtered to remove the brown precipitate. The filtrate is then added to the pyocine preparation. The slime forms a fluffy, white precipitate that is removed by centrifugation. This method has the advantage over that of Kageyama and Egami (1962) in that it is technically easier to perform. The efficacy of Brown's method of manganous chloride treatment was compared with that of DNase and acrinol and was found to be superior in slime removal. However, it was necessary to keep the final concentration of filtrate down to 4% to prevent diminution of pyocine activity.

Since pyocines are proteinaceous in nature, the established

methods of concentration and purification of proteins are applicable to pyocine preparations. Two methods of concentration were examined, both involving the removal of water and consequent reduction in volume of the pyocine preparation. The use of polyethylene glycol for concentrating fluids containing protein, reported by Kohn (1959), was found to be effective for concentrating pyocines, while retaining their activity. The rotary film evaporator allows the separation of solvents by distillation at a reduced pressure and controlled temperature. It operates on the principle of continuously spreading a thin film of solution over a large area, so providing a large evaporating surface. Since rotary film evaporation is particularly suitable for concentrating heat-sensitive solutes, its application to pyocine preparations was investigated. However, it was found to be unsatisfactory because the pyocine activity was destroyed or considerably reduced.

The disadvantage of both methods was that the impurities in the preparations were concentrated together with the pyocines; it might have been better to purify the pyocine preparations before trying to concentrate them by these methods.

Salting-out by saturation of a preparation with ammonium sulphate is an established technique for purifying proteins and can also be used as a method of concentration. The application of salting-out to pyocine preparations was reported by Hamon (1956) and has since been advocated by many authors (Kageyama and Egami,

1962; Higerd, Baechler and Berk, 1967; Takeya et al., 1969). The recommended degree of ammonium sulphate saturation varies from 70 - 80%. In this study, 75% saturation (i.e. addition of 50% w/v ammonium sulphate) was tested and found to be effective. The fluffy material that collects after ammonium sulphate treatment usually forms a precipitate which is sedimentable by centrifugation (Kageyama and Egami, 1962) but it may remain as a surface layer which is more difficult to collect (Takeya et al., 1969). In the three pyocine preparations tested, the protein formed a precipitate at 4°C and was collected by centrifugation in the cold. Concentration was effected by resuspending the precipitate in a small volume of buffer. Better results were obtained when the ammonium sulphate-treated pyocine was held at 4°C for 24 - 48 hours rather that the shorter time described by other workers (Kageyama, 1964; Higerd, Baechler and Berk, 1967).

Ultracentrifugation was found to be a useful method for concentrating the high molecular weight pyocines and also freed the preparation of low molecular weight contaminants. However, it was less effective than ammonium sulphate in concentrating the pyocines and was limited by the small volume that could be centrifuged at one time. When ultracentrifugation was used in sequence with ammonium sulphate treatment for the purification and concentration of high molecular weight pyocines, it gave very good results. However, ultracentrifugation is useless in the concentration of small pyocines, such as pyocine H108, because they are non-sedimentable.

The "shelf-life" of purified pyocines is relevant to their use as therapeutic agents because if preparations retained their activity on storage, large batches of pyocine could be made at one time and held until required. Kageyama and Egami (1962) found that the activity of a high molecular weight pyocine was completely destroyed after lyophilisation. Higerd, Baechler and Berk (1969) found that a contractile pyocine preparation lost its activity when preparations were frozen and thawed, but suspension in 12% dimethylsulphoxide or 5% glycerol, followed by storage at -60°C protected the protein and the pyocine retained its activity. These authors did not record the duration of storage. Colicines E_I and K have been reported to retain their activity after several rounds of freezing and thawing (Fields and Luria, 1969) and it was thought that the same finding might be true of small pyocines. Indeed, the small pyocine H108 was more active after storage at -20°C than at 4°C or 22°C. In contrast, the high molecular weight pyocines, 5893 and 5882, retained their activity better at 4°C. These pyocine preparations were stored without any protective agent because an earlier experiment had shown that it was difficult to separate the pyocines from glycerol after storage. In general, it was found that purified pyocines suffered a greater loss of activity after storage than the less pure preparations.

Pyocines are not generally thought to be toxic although Homma and Suzuki (1964) showed that some cell-wall associated proteins with pyocine activity were capable of eliciting a

Schwartzman phenomenon and a pyrogenic response. Higerd, Baechler and Berk (1967) found that a purified contractile pyocine preparation was not lethal to mice when injected intraperitoneally at levels of 28,000 - 1,400,000 units. Neither did these authors observe dermonecrosis when pyocines were given to rabbits subcutaneously. Govan (1968) found that the contractile pyocine R21 was non-toxic to mice in impure or purified forms (both at 400,000 units per ml), when administered by various routes. Before pyocines could be proposed as useful therapeutic agents, it was necessary to show that they were not toxic or lethal to animals.

The toxicities of preparations of pyocines 5893, 5882 and H108 at various stages of concentration and purification were examined. No adverse effects were observed in mice that received pyocine 5882, but pyocines 5893 and H108 did appear to have some toxic properties. The toxicity of the small pyocine H108 may be related to the observation by Homma and Suzuki (1964) concerning cell-wall associated pyocines.

Initially, it was thought that the lesions observed in mice injected subcutaneously with pyocines 5893 and H108 were the manifestation of local reactions to the toxic pyocine preparations. However, later experiments suggested that the fluid injected subcutaneously did not always disperse, but remained concentrated around the neck, thereby producing an irritation to which the

mouse reacted by scratching. In the light of these findings, when pyocine 1577 was tested for toxicity, the volume of the subcutaneous injections was reduced from 0.5 ml to 0.3 ml and the neck was massaged after injection. No lesions were observed in any of the later experiments.

In view of the apparent toxic effects of pyocine 5893, it was decided to examine preparations of this pyocine in greater detail. The findings of other workers suggest that the contractile pyocine itself is not toxic (Higerd, Baechler and Berk, 1967; Govan, 1968) and the adverse effects that were observed may have been caused by other substances present in the preparations. A comparative examination of the effects of uninduced and induced preparations of pyocine 5893 supported the theory that the pyocine was not the cause of the toxicity; there were no deaths among the mice injected with induced pyocine preparations, but 50% mortality among mice that received uninduced preparations, low in pyocine activity. This result suggests that extracellular products other than pyocines may be incriminated in toxicity and that more of these are present in the uninduced preparations.

Slime and extracellular enzymes as well as a protein exotoxin have been implicated in the pathogenicity of Ps. aeruginosa (Liu, Abe and Bates, 1961; Liu, 1966). Slime was removed from all the pyocine preparations before their toxicity was examined, but non-pyocine protein present in the pyocine-containing culture supernate was concentrated with the pyocine by polyethylene glycol

and ammonium sulphate treatment, and it was in mice that received such preparations that toxic effects were most frequently seen. Induced and uninduced stage II preparations of pyocine 5893 were examined for their haemolytic and extracellular enzyme activity by methods described by Liu et al. (1961). Most strains of Ps. aeruginosa produce a variety of extracellular enzymes but maximum production usually occurs after three or more days incubation of a culture. Govan (1968) found that preparations of pyocine R21 infrequently contained haemolysins, lecithinases or lipases, and gelatinase activity, if present, rarely exceeded a titre of 1 in 64. Preparations of pyocine 5893 tested in this study, were able to digest casein, suggesting the presence of a protease, but there was no lecithinase or haemolytic activity detectable. Liu and Mercer (1963) suggested that protease and lecithinase were the most important enzymes in the pathogenesis of pseudomonas infections but the lack of enzymatic activity in preparations of pyocine 5893 suggests that the enzymes were not the toxic agents.

In order to examine pyocine 5893 further, a concentrated preparation was fractionated by gel filtration, using Sepharose 4B (exclusion limits 10^5 - 2×10^7 molecular weight). This technique was applied to Ps. aeruginosa culture supernates by Carney and Jones (1968) and was found to separate extracellular enzymes from other protein material. These authors examined culture supernates up to seven days after inoculation, but made no mention of pyocine activity. Fractionation of an induced preparation of pyocine 5893 on Sepharose 4B resulted in two closely linked peaks

of pyocine activity, the second corresponding to the peak of ultra-violet-absorbing material. The elution of two pyocine peaks led to the suspicion that there was more than one kind of pyocine particle present. Elastase and protease activities of the eluate fractions were estimated by the methods described by Carney and Jones (1968). Although the level of enzyme activity was very low, the sample with maximum elastase activity was found to precede that of maximum protease activity. The latter corresponded to the peak of ultra-violet-absorbing material. When an uninduced preparation of pyocine 5893 was eluted from a Sepharose 4B column and the optical density of the eluate assayed, the peak of ultra-violet-absorbing material was found to occur somewhat later than the peak in the induced preparation. This may reflect different proteins in the two preparations.

When the toxicities of selected fractions of the induced pyocine 5893 preparation were tested in mice, the animals that received a sample of the first pyocine peak were the only ones that became visibly unwell. All the other groups of mice appeared healthy, although they did show slight loss in weight. This finding suggested that the pyocine itself, or some factor closely associated with it, was causing the toxic effects in mice.

In view of the undesirable findings associated with pyocine 5893; its toxicity, the possibility of production of more than one type of pyocine, and the generally low pyocine activity achieved even after induction and concentration, it was decided to resume the search for a contractile pyocine inhibitory to

Ps. aeruginosa strain P14. A further seventy-seven strains of Ps. aeruginosa were examined for pyocine production and strain 1577 was selected. Pyocine 1577 had the properties of a contractile pyocine when attacking indicator strain P14; it was poorly diffusible, resistant to the action of heat and trypsin, and sedimentable by ultracentrifugation. Electron-micrographs of pyocine preparations clearly revealed contractile particles attached to cells of strain P14. Injection of mice with preparations of pyocine 1577 at various stages of purification and concentration did not result in any deaths, but a considerable weight loss was recorded, particularly in animals that received the preparation with the greatest pyocine activity.

The adverse effects of pyocine preparations on mice were disturbing and it was decided to sacrifice a short preparative procedure for a more extended purification technique. This was based on that of Higerd, Baechler and Berk (1967) and is described in detail in the methods of this section. The buffer used throughout was one recommended in a later paper by these workers (Higerd, Baechler and Berk, 1969) and contained 0.02M-$MgCl_2$ which is reported to stabilise the contractile pyocines in the uncontracted, active state.

The purification procedure was lengthy, requiring at least ten days to complete. It was also limited by the volumes of pyocine that could be conveniently handled at any one time. In the initial stages, the slime was removed and the protein was collected by salting-out with ammonium sulphate. The high molecular weight protein was sedimented by ultracentrifugation

and a sample of the resuspended deposit was applied to a DEAE-cellulose ion-exchange column. When the pyocine was injected into mice before chromatography, it caused a considerable weight loss, but when eluate fractions, containing high levels of pyocine activity, were ultracentrifuged and the resuspended deposit was injected, the mice showed very little loss in weight. The pyocine activity of the final sample (stage VI) was higher than that of the intermediate sample (stage IV) which caused the greater weight loss. This suggests that the adverse effects are caused not by the pyocine, but by some factor closely associated with it, which is deposited in the initial ultracentrifugation but is separated from the pyocine by ion-exchange chromatography. With these findings in mind, it was decided that fully-purified pyocine should be used for all future in-vivo studies.

Chloroform is used routinely for sterilising bacteriocine preparations and was used in the early stage of this investigation, but it has been reported (Mayr-Harting, Hedges and Berkeley, 1972) that the potency of some bacteriocines is reduced by chloroform treatment. However, it was found to be unnecessary to use chloroform at later stages in the purification procedure because the pyocine preparation was free from Ps. aeruginosa contamination.

In order that the therapeutic use of the three selected pyocines should be comparable, the extended purification procedure was tested on pyocines 5882 and H108. Pyocine 5882 behaved similarly to pyocine 1577 and the peak elution of pyocine from the ion-exchange column was at approximately the same sodium

chloride concentration as that of pyocine 1577. When the optical density (at 280 nm) of the eluate samples of the two pyocines was compared, it was observed that pyocine 1577 had a protein peak which was eluted about 60 ml after the pyocine peak. This later peak was absent in pyocine 5882 elutions and this may be related to the complete lack of toxicity of this pyocine at all stages in its preparation.

The purification and concentration of pyocine H108 presented several problems. Small pyocines are non-sedimentable in the ultracentrifuge and precipitation with ammonium sulphate is an important method of concentration. When a sample of pyocine H108 was applied to a DEAE-cellulose column in the same way as high molecular weight pyocines, the results confirmed those of Ito, Kageyama and Egami (1970); i.e. the small pyocine did not absorb to DEAE-cellulose but passed through the column. These authors reported that chromatography with CM-cellulose resulted in a considerable loss of pyocine activity, but Ohkawa et al. (1973) found that while 95% of the ultra-violet-absorbing material passed through a CM-cellulose column, most of the small pyocine was absorbed and eluted by increasing the concentration of sodium chloride. When the method of Ohkawa et al. (1973) was applied to pyocine H108, the pyocine did not absorb to the resin but passed through the column in the preliminary buffer wash.

Small pyocines have been shown to be unstable at room

temperature and at low salt concentrations (Ohkawa et al., 1973) and are not generally produced in high titres even after induction of the pyocinogenic strain (Holloway, pers. comm.). Pyocine H108 also appeared to have some toxic properties. When the survey for another contractile pyocine was carried out, the producer strains were also examined for small pyocine activity. However, no strain was recognised as producing a small pyocine superior to pyocine H108. Therefore, pyocine H108 remained the representative small pyocine throughout the investigation.

In summary, methods of purification and concentration of the selected pyocines (5893, 5882 and H108) were examined and effects of these pyocines on mice were observed. Pyocine 5893 was found to have undesirable effects in vivo although these could not definitely be ascribed to the pyocine. Therefore this strain was replaced by Ps. aeruginosa strain 1577 which also produced contractile pyocines active against strain P14. An extended purification procedure was established which included ion-exchange chromatography of the pyocine preparations. This technique gave good results with the high molecular weight pyocines but the small pyocine proved more difficult to handle.

SECTION 3

A STUDY OF THE INTERACTIONS *IN VITRO* BETWEEN PYOCINES 1577, 5882 AND H108 AND CELLS OF *PSEUDOMONAS AERUGINOSA* STRAIN P14

SECTION 3

A study of the interactions in vitro between pyocines 1577, 5882 and H108 and cells of Pseudomonas aeruginosa strain P14

METHODS

General methods used throughout this series of experiments

(1) Preparation of Pseudomonas aeruginosa strain P14

The in-vitro experiments in this section were designed as models for later in-vivo experiments. Therefore, the suspension of sensitive cells of strain P14 was prepared in the same manner as an infective dose. A 100 ml volume of $NB_{20}S$ was inoculated with a single colony of strain P14 from a nutrient agar plate and incubated overnight in a 2 l flask in an orbital incubator at 37°C and 100 r.p.m.. A 20 ml volume of this culture was centrifuged at 1850g for 30 min. and the cellular deposit was resuspended in 40 ml of $NB_{20}S$. The cell suspension was held at 4°C (for not longer than 3 hours) and warmed to 37°C immediately prior to use.

(2) Preparation of the pyocines

Pyocines 1577, 5882 and H108 were prepared to stage II (see Section 2) and the activity and sterility of the preparations was confirmed before use. Those that were contaminated with Ps. aeruginosa were sterilised by the addition of 5% v/v chloroform, mixed well and allowed to stand for 10 min.. The pyocine preparation was then decanted.

(3) Viable count technique

Early studies showed that the Miles and Misra technique for viable counts (Miles and Misra, 1938) was not entirely satisfactory for Ps. aeruginosa because the organism has a tendency to form slightly spreading colonies. Therefore a spread-plate technique was employed throughout these experiments.

A viable count of a culture was made by preparing a series of tenfold dilutions of the culture in saline in 3 in. x ½ in. tubes, using 0.1 ml of culture in 0.9 ml of saline. A fresh, sterile Pasteur pipette was used for each transfer and an automatic syringe was used throughout. Volumes of 0.1 ml of suitable dilutions were delivered by pipette on to the surface of A9 agar plates and the fluid was spread over the entire surface of the plate using the shorter arm of a glass spreader. A separate spreader was used for each plate. Dilutions were plated in triplicate unless otherwise stated. The spread-plates were allowed to dry, inverted, and incubated at 37°C overnight.

This method of making viable counts gave reproducible results.

Colony counts were made with the aid of a digital display unit, at a dilution of the culture that gave between c. 100 and 600 colonies per plate.

(4) Mixing of P14 cells and pyocine and sampling of the mixture

Equal volumes of a P14 cell suspension and a pyocine preparation were mixed in 100 ml conical flasks and incubated in a waterbath at 37°C under agitation. A cell control and a

pyocine control, in which pyocine and cells respectively were replaced by saline, were also incubated. At each sampling time, 2 ml were withdrawn from the test flask and 2 ml from the cell control flask and these samples were centrifuged in 5 in. x 5/8 in. tubes at 1600g for 20 min.. The supernate from the test sample was decanted into a tube containing 0.2 ml of chloroform and was later titrated, together with a sample from the pyocine control, for pyocine activity against Ps. aeruginosa strain P14. The supernate from the cell control sample was discarded. The deposits from both test and cell control samples were each resuspended in 2 ml of saline and viable counts were made by the method described above.

The details of the sampling times are given individually for each experiment.

The following experiments were carried out:-

(a) The effect of each pyocine individually on cells of strain P14.

(b) The effect of a mixture of pyocines 1577 (contractile), 5882 (filamentous) and H108 (small) on cells of strain P14.

(c) The long-term survival of strain P14 in the presence of pyocine.

(d) The effect of repeatedly dosing a culture of strain P14 with pyocine.

(a) The effect of each pyocine individually on cells of Ps. aeruginosa strain P14

A 10 ml volume of pyocine 1577 was mixed with 10 ml of a

suspension of P14 cells at the start of the experiment. The cell control contained 10 ml volumes of P14 cells and saline, and the pyocine control contained 10 ml volumes of pyocine 1577 and saline.

The first sample was taken at the start of the experiment from the cell control only, to give the initial viable count. Thereafter, samples were taken from both test and control flasks at hourly intervals for 3 hours and treated as described in the general methods.

The experiment was repeated using pyocine 5882 and using pyocine H108.

(b) The effect of a mixture of pyocines 1577, 5882 and H108 on cells of Ps. aeruginosa strain P14

A 9 ml volume of a P14 cell suspension was mixed with a "pool" containing 3 ml of each pyocine preparation (1577, 5882 and H108). The cell control contained 9 ml of the P14 cell suspension and 9 ml of saline. The pooled pyocine control contained 3 ml of each pyocine mixed with 9 ml of saline, and individual pyocine controls contained 1 ml of each pyocine preparation and 5 ml of saline.

At the start of the experiment, a viable count was made on a sample from the cell control, after centrifugation, as described in the general methods. Simultaneously, the pyocines were added to the cell suspension and incubation commenced, under agitation, at 37°C in a waterbath.

Samples were taken at hourly intervals for 3 hours and used to prepare viable counts. The pyocine activity of the test and pyocine control samples was estimated by titration against strain P14.

(c) The long-term survival of cells of Ps. aeruginosa strain P14 in the presence of pyocine

A P14 cell suspension in 0.5 ml volumes was mixed with 5 ml of each pyocine preparation (1577, 5882 and H108) in 6 in. x 5/8 in. test tubes. The cell control contained 0.5 ml of the cell suspension and 5 ml of saline and the pyocine controls contained 5 ml of pyocine and 0.5 ml of saline. All the mixtures were incubated in a 37°C incubator without agitation.

A viable count was made of a sample of the cell control at the start of the experiment. Samples were taken from the test and control mixtures at 24, 72 and 144 hours after the start of the experiment. Viable counts of the test samples and the cell control samples were made directly (omitting the centrifugation step described in the general methods) and 0.5 ml of each test sample was added to 0.1 ml of chloroform prior to assay of the pyocine activity of the sample against strain P14. The pyocine controls were also titrated.

Representatives of the survivor colonies, which grew on the viable count plates, were pyocine typed, and examined for resistance to the pyocine with which they had been treated, by a cross-streaking technique. A tryptone soya blood agar (TSBA) plate was inoculated with the pyocine producer strain (1577, 5882

or H108) diametrically across the plate in a strip about 1.5 cm wide. The plate was incubated at 32°C overnight and the macroscopic bacterial growth was removed with a sterilised glass microscope slide. The plate was exposed to chloroform vapour for 15 min. and then left slightly open to allow the vapour to disperse. Loopfuls of four-hour NB cultures of the survivor colonies were streaked across the plate at right angles to the original inoculum and the plates were incubated overnight at 37°C. An inhibition zone was clearly demonstrable when the survivor colony culture was still sensitive to the pyocine.

The results from the cross-streaking technique were confirmed by spotting a neat and 1 in 10 dilution of the pyocine preparation on to a lawn of the survivor colony culture.

(d) <u>The effect of repeatedly dosing a culture of Ps. aeruginosa strain P14 with pyocine</u>

A 10 ml volume of pyocine 1577 was mixed with 10 ml of a suspension of P14 cells in a conical flask and incubated under agitation for 1 hour at 37°C in a waterbath. The cell control contained 10 ml of saline and 10 ml of the P14 cell suspension and was incubated under the same conditions. A viable count of the cell control was made at the start of the experiment. After 1 hour of incubation, 2 ml of test and control samples were withdrawn, centrifuged, and used to prepare viable counts. Simultaneously, the remainder of the mixture of pyocine and cells

was centrifuged at 1850g for 20 min. and the cellular deposit was resuspended in 18 ml of fresh pyocine 1577. The resuspended mixture was incubated under the same conditions for a further 30 min. when the sampling and centrifugation procedure was repeated, and the cellular deposit resuspended in 16 ml of fresh pyocine 1577. Incubation was continued for a further 30 min. before another sample was taken. A viable count was made on a sample of the cell control at each sampling time.

The flasks containing the remainder of the pyocine and cell mixture and the cell control were held in the incubator at 37°C and were sampled 24 hours after the start of the experiment.

The experiment was repeated using pyocine 5882 and using pyocine H108.

A selection of the survivor colonies were checked for pyocine resistance by the cross-streaking technique described previously.

<u>An investigation into the resistance of Ps. aeruginosa strain P14 to pyocine 5882</u>

In view of the results of the previous experiment, of testing survivor colonies for resistance to pyocine 5882, a more detailed study was carried out.

A NB culture of strain P14 was plated out on to NA to give single colonies and incubated at 37°C overnight. Thirty-six colonies were picked and inoculated into 2 ml volumes of NB,

incubated for 4 hours at 37°C and used to cross-streak a prepared TSBA plate which had previously been inoculated with strain 5882 (see page 159). The cross-streaked plates were incubated at 37°C overnight. The inhibition of the 36 cultures by pyocine 5882 was recorded. A small amount of the growth from each cross-streak was used to inoculate a 2 ml volume of NB that was then incubated at 37°C for 4 hours. From these cultures, lawns were prepared on A9 agar plates. Volumes of 0.02 ml from a series of doubling dilutions of pyocine 5882 were spotted on to each lawn and after the drops had dried, the plates were incubated at 37°C overnight.

The 36 cultures were also examined for their sensitivity to pyocines 1577 and H108 by the cross-streaking technique.

RESULTS

(a) The effect of pyocines 1577, 5882 and H108 individually on Ps. aeruginosa strain P14

A culture of strain P14 was mixed with pyocine 1577, 5882 or H108 and viable counts were made on test and control samples at hourly intervals. The average viable count per ml of sample was calculated from the counts from three plates and the results are shown in tables 16a, b and c and fig. 15.

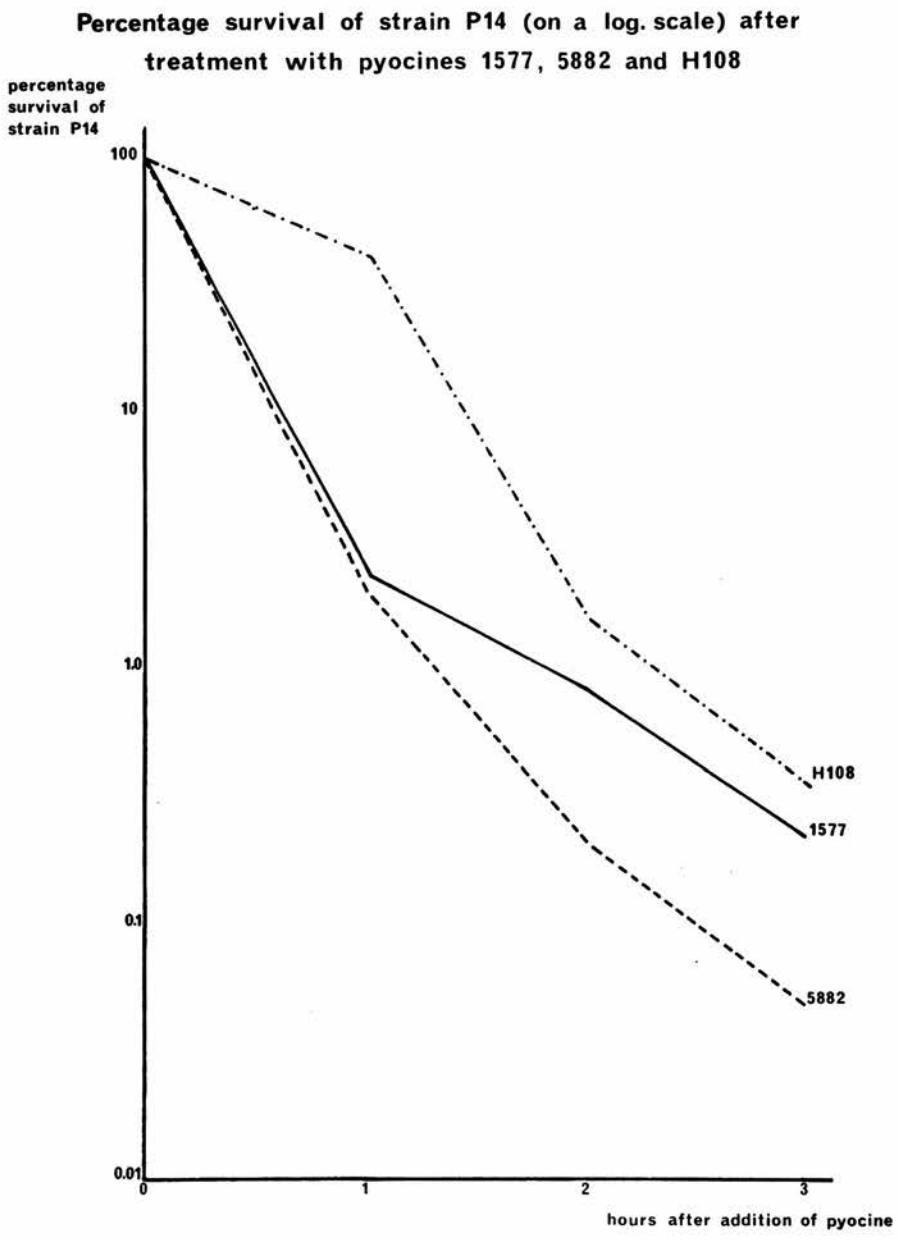
The three pyocine preparations appeared to reduce the viability of a P14 cell suspension by approximately the same amount i.e. by one hundred to one thousandfold, but the P14 population was never sterilised by these pyocine treatments.

During the three-hour incubation period, in the pyocine controls the activity of pyocines 5882 and H108 diminished, whereas that of pyocine 1577 was unchanged.

(b) The effect of a mixture of pyocines 1577, 5882 and H108 on a cell suspension of strain P14

The survival of a P14 suspension treated with a mixture of pyocines 1577, 5882 and H108, is shown in table 17. The activities of the individual pyocines against strain P14 before the start of the experiment were 100,000; 200,000; and 1600 units per ml respectively. The pool of three pyocines was titrated during the experiment and the results are included in table 17. Individual pyocine controls were also incubated and

FIGURE 15


Percentage survival of strain P14 (on a log. scale) after treatment with pyocines 1577, 5882 and H108
percentage survival of strain P14
100
10
1.0
0.1
0.01
0
1
2
3
hours after addition of pyocine
H108
1577
5882

titrated against strain P14 at hourly intervals. The activity of the pyocine 1577 control remained constant at 6400 units per ml throughout the experiment. The activity of the pyocine 5882 control dropped from 12,800 units per ml after 1 hour, to 6400 units per ml after 2 hours and to 3200 units per ml after 3 hours incubation. The pyocine H108 control showed no activity at all after 1, 2 or 3 hours incubation.

TABLE 16a The survival of strain P14 after treatment with pyocine 1577

Sample	Time of sampling (hours from start)	Pyocine activity against strain P14 (units per ml)	Average viable count of sample (cells per ml)	% survival
C	0	. . .	1.77×10^8	100
P	0	12,800	. . .	
C	1	. . .	9.4×10^7	
T	1	25,000	2.2×10^6	2.3
C	2	. . .	1.67×10^8	
T	2	25,000	1.43×10^6	0.86
P	2	12,800	. . .	
C	3	. . .	2.38×10^8	
T	3	25,000	5.33×10^5	0.23
P	3	12,800	. . .	

Key: C = cell control sample containing P14 cells and saline

P = pyocine control sample containing pyocine and saline

T = test sample containing P14 cells and pyocine

. . . = not performed

TABLE 16b The survival of strain P14 after treatment with pyocine 5882

Sample	Time of sampling (hours from start)	Pyocine activity against strain P14 (units per ml)	Average viable count of sample (cells per ml)	% survival
C	0	. . .	4.0×10^7	100
P	0	12,800	. . .	
C	1	. . .	4.4×10^7	
T	1	1600	8.8×10^5	2.0
P	1	12,800	. . .	
C	2	. . .	9.4×10^7	
T	2	800	2.0×10^5	0.21
P	2	6400	. . .	
C	3	. . .	1.2×10^8	
T	3	0	6.0×10^5	0.05
P	3	6400	. . .	

TABLE 16c The survival of strain P14 after treatment with pyocine H108

Sample	Time of sampling (hours from start)	Pyocine activity against strain P14 (units per ml)	Average viable count of sample (cells per ml)	% survival
C	0	. . .	5.24×10^7	100
P	0	800	. . .	
C	1	. . .	7.69×10^7	
T	1	800	3.24×10^7	40
P	1	400	. . .	
C	2	. . .	1.35×10^8	
T	2	400	2.15×10^6	1.6
P	2	200	. . .	
C	3	. . .	1.98×10^8	
T	3	200	7.19×10^5	0.4
P	3	200	. . .	

Key for both tables:

C = cell control sample containing P14 cells and saline

P = pyocine control sample containing pyocine and saline

T = test sample containing P14 cells and pyocine

. . . = not performed

TABLE 17 The survival of a P14 cell suspension treated with a pool of pyocines 1577, 5882 and H108 at the start of the experiment

Sample	Time of sampling (hours from start)	Activity of pooled pyocine against strain P14 (units per ml)	Average viable count of sample (cells per ml)	% survival
C	0	. . .	5.96×10^7	
P	0	50,000	. . .	100
T	0	. . .	. . .	
C	1	. . .	4.36×10^7	
P	1	25,000	. . .	15.1
T	1	. . .	6.59×10^6	
C	2	. . .	8.03×10^7	
P	2	25,000	. . .	5.0
T	2	12,800	4.04×10^6	
C	3	. . .	7.29×10^7	
P	3	25,000	. . .	7.0
T	3	12,800	5.10×10^6	

Key: C = cell control containing P14 cells and saline

P = pooled pyocine control containing pyocines 1577, 5882 and H108, and saline

T = P14 cell suspension + pooled pyocine

. . . = test not performed

(c) The long-term survival of cells of strain P14 in the presence of pyocine

Viable counts of P14 suspensions mixed with pyocines 1577, 5882 or H108 and incubated for 144 hours are shown in table 18, and the changes in the activities of the pyocines in table 19.

The sensitivity of the survivor colonies to the pyocine with which they were treated was examined by a cross-streaking technique and by spotting preparations of the pyocines on to lawns of the survivor colony cultures. After 24-hours incubation, a culture from the one surviving colony from the P14 suspension treated with pyocine 1577 was resistant to that pyocine when tested by cross-streaking. Cultures from six of the survivor colonies from a P14 cell suspension treated with pyocine 5882 were resistant to that pyocine, whereas six survivor colonies from pyocine H108 treatment remained sensitive to pyocine H108. After 72 hours incubation, a further selection of survivor colonies was tested by cross-streaking and again the survivors of pyocine 1577 and 5882 treatment were resistant to their respective pyocines but the survivors of pyocine H108 treatment were now resistant to pyocine H108.

The survivor colonies that were picked after 72 hours incubation were used to make lawns on to which drops of neat and 1 in 10 dilutions of the pyocine preparations were spotted. The results are shown in table 20.

TABLE 18 The long-term survival of cells of Ps. aeruginosa strain P14 after treatment with pyocines 1577, 5882 and H108

Sampling time (hours from start)	Average viable count of sample (cells per ml)			
	P14 cell control	1577 + P14 cells	5882 + P14 cells	H108 + P14 cells
0	2.2×10^7	. . .	. . .	. . .
24	3.8×10^7	*	6.5×10^4	1.8×10^4
72	6.6×10^7	5.7×10^7	9.3×10^7	8.8×10^7
144	3.8×10^7	2.9×10^7	4.6×10^7	4.3×10^7

Key: 1577 = pyocine 1577
5882 = pyocine 5882
H108 = pyocine H108
* = 1 colony was recorded on one plate from an undiluted sample
. . . = not tested

TABLE 19 The inhibitory activity of pyocines 1577, 5882 and H108 against Ps. aeruginosa strain P14 after 24, 72 and 144 hours incubation

Pyocine preparation	Activity of pyocine against strain P14 (units per ml) after:			
	0 hours	24 hours	72 hours	144 hours
1577 C	50,000-100,000	100,000	50,000	12,800
1577 T	. . .	100,000	25,000-50,000	12,800-25,000
5882 C	200,000	25,000-50,000	800	400
5882 T	. . .	6400-12,800	400-800	0
H108 C	1600	800	400	400
H108 T	. . .	400-800	0	0

Key: C = pyocine control containing pyocine and saline
T = pyocine + P14 cell suspension
. . . = not tested

TABLE 20 The pyocine resistance of survivors of strain P14, 72 hours after treatment with pyocines 1577, 5882 or H108

Pyocine used for treatment	Number of survivor colonies tested	Inhibition of lawn by pyocine:					
		1577		5882		H108	
		N	1/10	N	1/10	N	1/10
1577	15	-	-	+	+	+	+
5882	9	+	+	-	-	+	+
H108	15	+	+	+	+	-	-
...	P14 controls 15	+	+	+	+	+	+

Key: + = inhibition of lawn culture by pyocine

\- = no inhibition of lawn culture by pyocine

N = undiluted pyocine preparation

1/10 = one in ten dilution of pyocine preparation

(d) The effect of repeatedly dosing a culture of strain P14 with pyocine

Over a three-hour incubation period, a cell suspension of strain P14 was treated three times with a pyocine preparation and the results of the viable counts are shown in table 21, and represented graphically in fig. 16.

The activities of the pyocine preparations 1577, 5882 and H108 were 800,000; 200,000; and 3200 units per ml respectively when they were assayed against strain P14.

A selection of the survivor colonies were tested by cross-streaking, for resistance to the pyocine with which they had been treated. The survivors of pyocine 1577 and pyocine H108 treatment retained their sensitivity to those pyocines, whereas 50% of the survivors of pyocine 5882 treatment were no longer sensitive to pyocine 5882. This result was confirmed by titrating pyocine 5882 against lawns of cultures of these survivor colonies.

An investigation into the resistance of strain P14 to pyocine 5882

Thirty-six colonies of strain P14 that had never encountered pyocine 5882 were inoculated into NB and the sensitivity of the resulting cultures to pyocine 5882 was tested by cross-streaking. The type of colonial growth was also recorded.

Twenty of the 36 cultures were inhibited by pyocine 5882, whereas 16 cultures were resistant to the pyocine. All the cultures that were inhibited by pyocine 5882 had a smooth

TABLE 21 Survival of a culture of strain P14 after repeated treatment with pyocines 1577, 5882 or H108

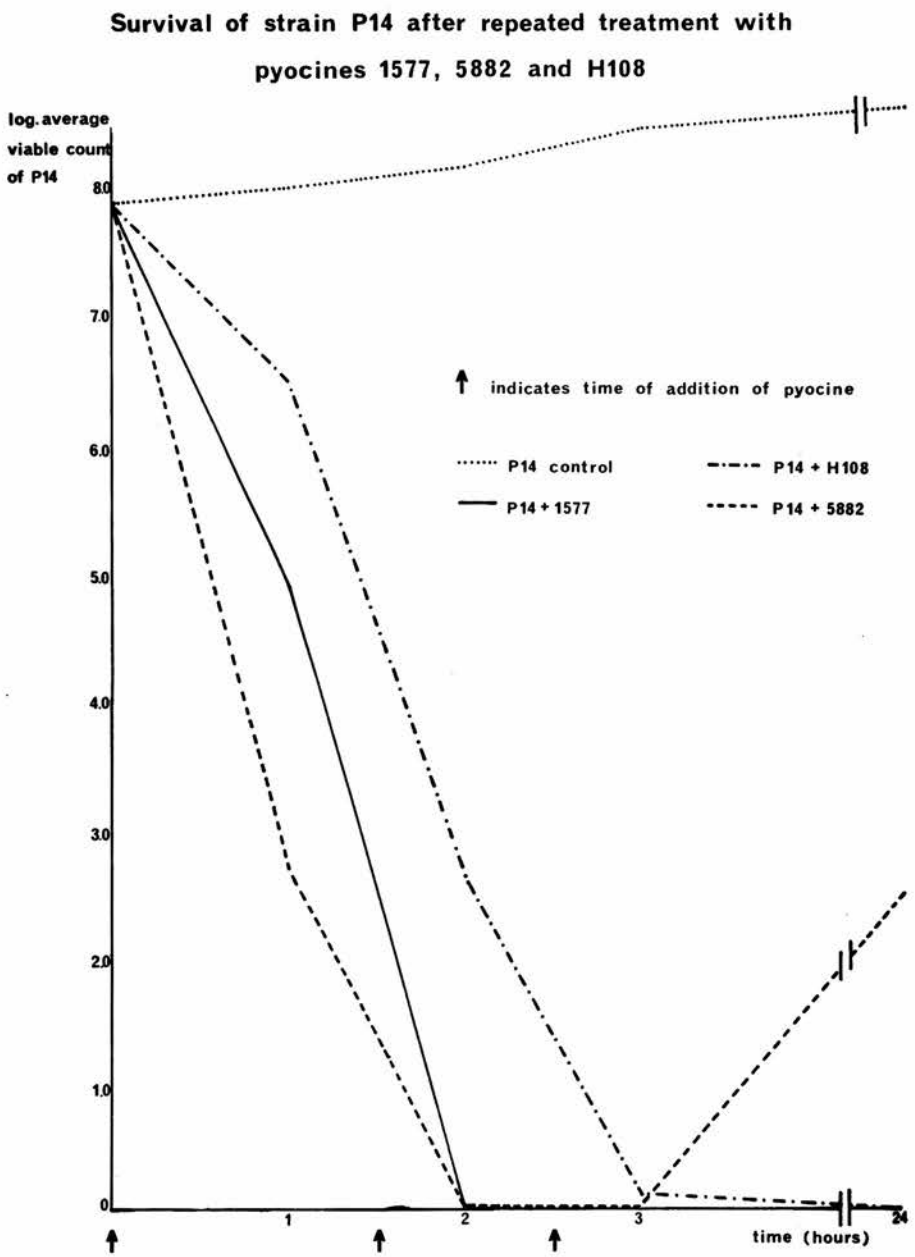
Pyocine	Sample	Time of sampling (hours from start)	Addition of pyocine dose (hours from start)	Average viable count of sample (cells per ml)
1577	C	0		1.2×10^8
	T		0	
"	C	1		8.4×10^7
"	T	1		7.4×10^4
			1.5	
"	C	2		1.7×10^8
"	T	2		0
			2.5	
"	C	3		1.9×10^8
"	T	3		0
"	C	24		3.8×10^8
"	T	24		0
5882	C	0		8.2×10^7
	T		0	
"	C	1		8.5×10^7
"	T	1		4.6×10^2
			1.5	
"	C	2		1.4×10^8
"	T	2		0
			2.5	
"	C	3		2.7×10^8
"	T	3		*
"	C	24		3.9×10^8
"	T	24		6.3×10^2
H108	C	0		8.3×10^7
	T		0	
"	C	1		7.5×10^7
"	T	1		2.7×10^6
			1.5	
"	C	2		1.8×10^8
"	T	2		3.3×10^2
			2.5	
"	C	3		1.8×10^8
"	T	3		2.3×10
"	C	24		2.2×10^8
"	T	24		0

* 3 colonies were isolated on one plate

Key: C = cell control containing P14 cells and saline

T = P14 cell suspension and pyocine

FIGURE 16


Survival of strain P14 after repeated treatment with pyocines 1577, 5882 and H108
log. average viable count of P14
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
1
2
3
24
time (hours)
indicates time of addition of pyocine
P14 control
P14 + H108
P14 + 1577
P14 + 5882

colonial morphology whereas 12 of the resistant cultures had a rough colonial morphology and the remaining four resistant cultures had a smooth colony type.

The results of the cross-streaking were confirmed by using the cultures to make lawns on which pyocine 5882 was titrated. It was found that either the culture was completely resistant to pyocine 5882 or else it was sensitive to 25,000 units per ml of the pyocine.

When the cross-streaking procedure was repeated and the 36 cultures were examined for their sensitivity to pyocines 1577 and H108 as well as 5882, they were all still inhibited by pyocine H108. Of the 16 cultures that were resistant to pyocine 5882, six were also resistant to pyocine 1577, while the other 10 remained sensitive to pyocine 1577. Those cultures that were resistant to both pyocines 1577 and 5882 had a rough colonial form, but of those cultures that were resistant only to pyocine 5882, 50% were rough and 50% were smooth colony types.

DISCUSSION

Studies on the mode of action of bacteriocines have been confined mainly to colicines but it is thought that at least some pyocines act in the same general manner. The evidence suggests that a single bacteriocine molecule is capable of killing a cell by a two-stage process. First, the bacteriocine adsorbs to a specific receptor site on the bacterial cell surface; this stage is reversible and the bacteriocine may dissociate or it may proceed to the second stage and kill the cell. Some contractile pyocines (Kageyama, Ikeda and Egami, 1964) and a small pyocine S2 (Ohkawa, Kageyama and Egami, 1973) have been shown to act by this single-hit killing process. The mode of action of filamentous pyocines has not been described. It is thought that killing begins as soon as the pyocine is added to a sensitive bacterial culture (Reeves, 1965) and the survival of the bacteria is related to the dose of pyocine administered (Kageyama et al., 1964).

The method of assay of pyocine activity used throughout this study has been the critical dilution method i.e. the estimation of the dilution at which a pyocine preparation is still capable of inhibiting the growth of a sensitive indicator lawn. This method is limited by the difficulty in accurately determining the end-point, but because of its extreme simplicity it has proved very useful. The results are expressed in arbitrary units per ml of a pyocine preparation (units per ml = 50 x reciprocal titre of activity) but this does not measure the lethality of the pyocine

preparation. A survivor count method of estimating pyocine activity can lead to the expression of activity in lethal units per ml (Mayr-Harting, Hedges and Berkeley, 1972).

Important features of the survivor count method include the rapid and thorough mixing of pyocine preparation and sensitive cells and continued agitation throughout the reaction period. Although incubation was carried out under agitation in all but one of the experiments described in this section, a comparative study suggested that the lethal effect of pyocine 5882 was just as great under static incubation conditions as it was under continuous agitation.

It is also important (Mayr-Harting et al., 1972) that a sample of the reaction mixture is diluted immediately after it is taken, to prevent further adsorption of the pyocine particles to the cells. In preliminary studies, it was found that a 1 in 10, or 1 in 100 dilution of the cells and pyocine mixture did not prevent the transfer of active pyocine on to the agar plates and killing of survivors during the incubation of the plate. Thus the results appeared to be anomalous, with higher viable counts at higher dilutions. To circumvent this problem, samples of the reaction mixture were centrifuged to deposit the surviving cells and the pyocine-containing supernate was decanted. The cellular deposit was resuspended in saline and a viable count was made. By this method, only the pyocine particles already attached to the cells were sedimented. It was felt that the introduction of a centrifugation step may have reduced the survivor count slightly,

due to loss of cells that were inadvertantly discarded with the supernate. But a comparative study showed that there was very little difference between the viable counts of centrifuged and uncentrifuged control samples. An alternative method would have been the addition of specific anti-pyocine antiserum to the sample to inactivate the pyocine. This method was not used because it would have necessitated large volumes of antisera and assay of the neutralising power of the antisera before each experiment.

The technique for making viable counts was studied in some detail to ensure that the method gave reproducible results. Automatic syringes tend to be somewhat inaccurate but they were used throughout, which helped to keep the inaccuracy constant and they made viable counts considerably easier to prepare than when using calibrated glass pipettes. A comparison of Miles and Misra and spread-plate techniques for viable counts revealed that the former resulted in lower counts, probably because the bacterial colonies tended to merge and become difficult to count accurately. Therefore the spread-plate technique was used throughout. Further investigations showed that it was essential to use a different Pasteur pipette for each transfer of fluid in making the dilutions, to prevent carry-over of excess culture from one dilution to the next.

The viable count of the P14 cultures used in these experiments was approximately the same as that of an infective dose for mice in order that these results could be related to later in-vivo studies.

In this section, the survival of cells of strain P14 was examined after treatment with the three different kinds of pyocines; 1577 (contractile), 5882 (filamentous), and H108 (small). When each pyocine preparation was mixed individually with a culture of strain P14 and survival examined over a three-hour period, pyocine 5882 was found to kill a greater percentage of the P14 cells than either of the pyocines 1577 or H108. There was a 0.05% survival rate after pyocine 5882 treatment, compared with 0.2% and 0.4% after pyocine 1577 and pyocine H108 treatments respectively. Thus, although pyocines 5882 and 1577 had the same initial activity (expressed in arbitrary units), the killing potential of the latter was lower or there was a greater number of the P14 population resistant to pyocine 1577 than to pyocine 5882. Although the initial activity of pyocine H108 (in arbitrary units) was only one sixteenth of that of the high molecular weight pyocines, its killing capacity was half that of pyocine 1577. The reason for this has still to be elucidated.

The rate of reduction in the viability of the P14 culture after pyocine treatment was slower than that reported by other workers. Kageyama et al. (1964) found that the killing activity of a contractile pyocine preparation was completed within about 20 min. whereas in these experiments the killing continued for at least 3 hours.

The P14 cultures were not sterilised by pyocine treatment even though there was active pyocine remaining in the mixture after 3 hours. Although it is thought that only one pyocine particle

is required to kill a cell, the pyocine must first collide with and adsorb to the cell surface. When there are relatively few cells and pyocine particles, the chances of collision are considerably reduced and this may explain how some cells escape death. When a preparation of pyocine 1577 of much higher activity (6,400,000 units per ml) was mixed with a culture of strain P14, the survival rate was reduced to c. 3×10^{-5}%. Pyocine resistance also has a role to play in the survival of P14 cells and this is discussed later.

The effect of treating a P14 culture with a mixture of pyocines 1577, 5882 and H108 was disappointing in that there was a higher percentage survival of P14 than resulted from the individual pyocine treatments. This may have been due to inactivation of one of the pyocines by another in the pyocine pool but was difficult to ascertain since the individual activities of the component pyocines of the pool could not be assessed. The pool of pyocines could have been titrated against three different indicator strains, each of which was only sensitive to one of the pyocines, but it would have been unwarranted to assume that this activity was the same as the activity of the pyocine against strain P14.

The receptors for some contractile pyocines are known to reside in the lipopolysaccharide (LPS) of the cell walls of sensitive cells (Ikeda and Egami, 1969; Stewart and Young, 1971), whereas the receptor sites for filamentous and small pyocines have

not been described. It was hoped that by treating a population of P14 cells with a mixture of the different kinds of pyocines, those cells that escaped attack by one kind might succumb to another. Also, the cells are less likely to develop resistance to all three types of pyocine simultaneously.

Pyocine resistance, or tolerance, appeared to play an important role in the long-term survival of P14 cultures in the presence of pyocine. Within 24 hours of treatment of such a culture with pyocine 1577, there was a dramatic reduction in the viability of strain P14, but a culture from the one surviving colony was found to be resistant to pyocine 1577. From a small number of pyocine-resistant cells at 24 hours, the P14 population had returned to its initial level within 144 hours of the start of the experiment and all the survivors tested were resistant to the pyocine.

Treatment of a culture of strain P14 with pyocine 5882 was apparently less effective than pyocine 1577 within 24 hours, or alternatively, the population of pyocine 5882-resistant variants had started to increase within the 24 hour period. Whereas the activity of pyocine 1577 only dropped slightly during 144 hours incubation at 37°C, the activity of pyocine 5882 was reduced in the pyocine control and absent in the P14 and pyocine mixture by the end of the experiment. This may be related to the heat lability of pyocine 5882 described in section 1.

The pattern of survival of strain P14 in the presence of pyocine H108 was very similar to that of pyocine 5882, but pyocine-resistant variants of P14 were not observed until 72 hours

after commencement. By this time, there was no pyocine H108 activity remaining in the mixture and the combination of this and the resistant variants probably contributed to the subsequent rise in the survivor counts.

The most effective way of reducing the viability of a population of P14 cells was by repeatedly treating the culture with pyocine over a 3-hour incubation period. Three treatments with pyocines 1577 or H108 eliminated the population of P14 cells within 24 hours. No P14 survivors were observed after three treatments with pyocine 5882 in 3 hours, but when the mixture was sampled after 24 hours, a significant number of P14 cells were recovered. A selection of the survivors were tested for their resistance to pyocine 5882. Most of those isolated within the first 3 hours incubation were sensitive to the pyocine, but all of those isolated after 24 hours were found to be resistant to pyocine 5882.

In view of the rapid emergence of resistance to pyocine 5882 among P14 cells, a population of strain P14 that had not previously encountered pyocine 5882 was examined. Sixteen of the 36 cultures grown from single colonies of strain P14 were found to be completely resistant to pyocine 5882 and six of these were also resistant to pyocine 1577. Those cultures that were resistant to both pyocines had a rough colonial morphology. Dissociation is a common phenomenon in Ps. aeruginosa and Homma (1971) reported differences in pyocine sensitivity between different

colony types of Ps. aeruginosa. In salmonellae, rough mutants are deficient in the side-chains that constitute part of the cell wall LPS. The rough variants of strain P14 may have been naturally-occurring mutants that had an altered cell wall LPS and therefore altered pyocine receptor sites. Although it is not known whether filamentous pyocines bind to the LPS, a change in the conformation of the LPS could affect the ability of the pyocines to bind to their receptor sites.

In summary, it was possible to reduce, but not to eliminate, the organisms in a P14 culture by treatment with pyocines 1577, 5882 or H108. Results were improved when highly active pyocine preparations were used. Resistance or tolerance to pyocine attack emerged rapidly and it was found that a proportion of a P14 culture was naturally resistant to pyocines 1577 and 5882. The development of resistance may hinder the successful use of pyocines as therapeutic agents.

SECTION 4

AN INVESTIGATION INTO THE POSSIBLE THERAPEUTIC USE OF PYOCINES IN *PSEUDOMONAS AERUGINOSA* INFECTIONS

SECTION 4

An investigation into the possible therapeutic use of pyocines in Pseudomonas aeruginosa infections

METHODS

Preparation of the infective dose of Pseudomonas aeruginosa

Pseudomonas aeruginosa P14 is a mouse-virulent strain which is lethal to the animals when administered in adequate doses. Previous workers have shown that even with virulent strains of Ps. aeruginosa, a large dose of organisms is required to produce mortality following intraperitoneal administration (Bartell, Orr and Garcia, 1968). In the present work, a number of pilot experiments showed that a suitable infective dose of strain P14 could be prepared in the following manner:-

100 ml of 20% nutrient broth in saline ($NB_{20}S$) were inoculated with a single colony of P14 from a nutrient agar (NA) plate and incubated in a 2 l flask in an orbital incubator at 37°C and 100 r.p.m. overnight. A 20 ml volume of this culture was then centrifuged at 1850 g for 30 min. and the cellular deposit resuspended in 50 ml of $NB_{20}S$. Viable counts of cell suspensions prepared in this way were in the range $1.0 - 3.0 \times 10^8$ organisms per ml. A 0.5 ml volume of such a suspension injected intraperitoneally into mice caused 100% mortality in 6-48 hours.

This standard method was used for the preparation of the

infective dose of strain P14 in subsequent experiments unless otherwise stated.

Activity of pyocines in vivo

In-vitro experiments have shown that treatment of a P14 culture with pyocine preparations 1577, 5882 or H108 resulted in a significant reduction in the viability of the culture. Prior to examining similar treatments in vivo it was necessary to show that the pyocine preparations were not inactivated by the body fluids of the animal. Braude and Siemienski (1965) showed that following subcutaneous injection of colicine into mice, inhibitory activity against a colicine-sensitive strain could be found in the sera. Mouse serum is not normally bactericidal thus the inhibitory activity was thought to be due to colicine activity. The aim of the following experiment was to examine the inhibitory activity of pyocines in the sera of mice that had been injected by different routes.

Stage II preparations of pyocines 1577, (8.0×10^5 units per ml), 5882 (4.0×10^5 units per ml), and H108 (3.2×10^2 units per ml) were used. Groups of five mice (CFE, ♀) were inoculated with 0.3 ml volumes of pyocine intravenously, intraperitoneally or subcutaneously. Control mice received sterile SGB by the same routes. One mouse from each group was killed by ether at 0.5, 1, 3, 5 and 24 hours after inoculation and bled from the posterior vena cava. The blood was allowed to clot, the serum was removed and tested for inhibitory activity against Ps. aeruginosa strain P14. Lawns of P14 were prepared on A9 agar plates and

0.02 ml drops of doubling dilutions of each serum in saline were applied to the lawns. The drops were allowed to dry and the plates were incubated at 37°C overnight. The inhibitory activity of each serum was recorded.

The effect of pyocines on Ps. aeruginosa in vivo

(1) By the intraperitoneal route

(A) Using pyocine 1577

Pyocine 1577 was prepared and purified to stage VI and suspended in THM buffer.

The infective dose of Ps. aeruginosa strain P14 was prepared by the standard method. Viable counts of the cell suspension were made before the first and after the last injection and the infective dose was calculated from the average of these counts. The cell suspension and the pyocine were held at 4°C between injection times and allowed to warm to room temperature (c. 22°C) immediately prior to injection.

Female CBA mice were distributed at random into groups of six and caged in these groups. The animals were given food and water ad lib.. Each mouse received two 0.5 ml intraperitoneal injections on day 1. The injection schedule is given in table 22.

TABLE 22 Injection schedule for experiment (A)

Group number	Time of injection (hours)		
	0	3	6
1	P14 1577*	-	-
2	P14	1577	-
3	P14	-	1577
4	P14	THM buffer	-
5	1577	P14	-
6	1577	-	P14
7	1577	THM buffer	-
8	$NB_{20}S$	$NB_{20}S$	-

Key:

* Mice in Group 1 were all injected with strain P14 into one side of the peritoneal cavity and then with pyocine 1577 into the other side.

THM buffer = 0.02M-Tris HCl + 0.02M-$MgCl_2$ buffer pH 7.6

$NB_{20}S$ = 20% nutrient broth in saline

P14 = Ps. aeruginosa strain P14; cells suspended in $NB_{20}S$

1577 = Pyocine 1577; suspended in THM buffer

\- = No injection

The mice were weighed before injection and daily thereafter for 13 days. The spleens were removed from the mice that died during the course of the experiment and were examined for Ps. aeruginosa by homogenising in 2 ml of saline. The homogenate was inoculated on to blood agar and cetrimide agar and any Ps. aeruginosa isolated were pyocine typed. At the end of the

experiment the surviving mice were killed by ether and bled from the posterior vena cava. The blood from mice in the same group was pooled, allowed to clot, and the serum was removed and stored at 0°C prior to serological investigations. The spleens were also removed from these mice and treated as described above.

(B) Using pyocine 5882

The above experiment was repeated with pyocine 5882. Pyocine 5882 and the infective dose of Ps. aeruginosa strain P14 were prepared by the methods used in experiment (A). In addition, the effect of pyocine on infection caused by a pyocine 5882-resistant variant of strain P14 was examined. P14/R was a mutant of strain P14 that was not inhibited by pyocine 5882 in vitro. The infective dose of strain P14/R was prepared by the same method as that for P14.

CBA mice of both sexes were used (adequate numbers of mice of one sex were not available at the time). The mice were caged in groups of six; each group being composed of mice of the same sex. On the first day of the experiment each mouse received two 0.5 ml injections intraperitoneally. The injection schedule is given in table 23. The mice were weighed and observed as described in experiment (A).

TABLE 23 Injection schedule for experiment (B)

Group number	Time of injection (hours) 0	3	6
1	P14 5882*	-	-
2	P14	5882	-
3	P14	-	5882
4	5882	P14	-
5	5882	-	P14
6	P14/R 5882*	-	-
7	P14/R	5882	-
8	P14/R	-	5882
9	5882	P14/R	-
10	5882	-	P14/R
11	P14	THM buffer	-
12	P14/R	THM buffer	-
13	5882	THM buffer	-
14	$NB_{20}S$	$NB_{20}S$	-

Key:

* Mice in groups 1 and 6 were all injected with strain P14 or P14/R into one side of the peritoneal cavity and then with pyocine 5882 into the other side

THM buffer = 0.02M-Tris HCl + 0.02M-$MgCl_2$ buffer pH 7.6

$NB_{20}S$ = 20% nutrient broth in saline

P14 = Ps. aeruginosa strain P14 suspended in $NB_{20}S$

P14/R = Ps. aeruginosa strain P14/R (pyocine 5882-resistant) suspended in $NB_{20}S$

5882 = Pyocine 5882 suspended in THM buffer

\- = No injection

(C) Using pyocine H108

It was not possible to obtain a highly active suspension of pyocine H108 because of the problems that have been discussed

previously. The effect of pyocine H108 on infections caused by Ps. aeruginosa strain P14 in mice was studied.

Pyocine H108 was prepared and purified and applied to a DEAE-cellulose column as described in section 2. The pyocine did not adsorb to the column but was washed through with buffer. This wash was collected and used for the experiment.

The infective dose of Ps. aeruginosa strain P14 was prepared by the standard method.

Three groups of four CFE mice were used. Group 1 was injected with 0.5 ml of P14 cell suspension; group 2 received 0.5 ml of pyocine H108 and group 3 received 0.5 ml of P14 followed by 0.5 ml of pyocine H108. All injections were intraperitoneal.

The effect of pyocines on Pseudomonas aeruginosa in vivo

(2) Infection of burns with Ps. aeruginosa

The methods used for burning and infecting mice were based on those described by Jones, Jackson and Lowbury (1966). Male hairless mice were used throughout. Each mouse was kept in an individual cage which had been sterilised before the start of the experiment. The sawdust bedding was replaced by sterile paper towels which were changed daily. The mice received food and water ad lib..

Burning:- The mice were anaesthetised by intraperitoneal injection of Nembutal solution (1 part concentrated Nembutal to 9 parts saline given at 0.1 ml per g body weight). A burn was

made on the back of each mouse by the application for 10 s of a standard brass block heated to 100°C in boiling water. This resulted in a full skin thickness burn of an estimated 6% of the body surface (Jones et al., 1966).

Infection:- The infective dose of Ps. aeruginosa strain P14 was prepared by the standard method described previously. Three hours after burning, the burn on each mouse was inoculated with 0.1 ml of a P14 cell suspension delivered by means of a Pasteur pipette. The bacteria were spread over the burned area with a sterile bacteriological loop. Uninfected control animals were inoculated with $NB_{20}S$ only.

Treatment:- This is described in the details of each experiment.

Experiment 1 Infection of burns with Ps. aeruginosa strain P14

Eight mice were burned. Four of the burned animals were infected with strain P14 and the remainder of the mice were not infected. The animals were observed for 18 days and swabs of the burns were taken on the third and seventh day.

Experiment 2 Treatment of infected burns with stage VI pyocine 1577

Twenty-four mice were arranged in groups of four (although each mouse was caged individually). The mice were burned and infected as described. A schedule of infection and treatment is given in table 24. Stage VI pyocine 1577 was used for treating the burn infections. Topical pyocine treatment was effected in the first instance by pipetting 0.4 ml of pyocine 1577 onto the

burn and spreading it with a wire loop. But it was found that most of the fluid ran off the burn, so the method was altered and a sterile cotton-wool swab soaked in pyocine was rubbed over the whole of the burned area. A fresh swab was used for each mouse. This method was found to be more satisfactory and was used hereafter. Topical treatment was given 3 times daily at 2 hour intervals on days 2, 3 and 4 of the experiment.

Systemic pyocine treatment was given by intravenous inoculation of a 0.3 ml volume of pyocine 1577 into the tail vein, once daily on days 2, 3 and 4 of the experiment.

TABLE 24 Schedule of infection and treatment of mice in experiment 2

Group number	Infection with strain P14	Treatment with pyocine 1577	
		Topical	Systemic
1	+	+	-
2	+	-	+
3	+	-	-
4	-	-	-
5	-	+	-
6	-	-	+

Key:

+ = regime followed

- = regime not followed

The mice were weighed and observed daily for 14 days. The bacterial flora of the burns was monitored before and after infection by frequent swabbing with cotton-wool swabs moistened

in sterile saline. The swabs were cultured on MacConkey agar and any Ps. aeruginosa isolated were pyocine typed. The spleens were removed from any mice that died during the experiment. Each spleen was homogenised in 2 ml saline and the homogenate plated on MacConkey agar.

At the end of the experiment the state of each burn was recorded, the mice were killed by ether and bled from the posterior vena cava. The blood was allowed to clot and serum removed and stored at 0°C prior to serological investigations.

Experiment 3 Treatment of burn infections with stage IV and stage VI preparations of pyocine 1577

Purified (stage VI) pyocine preparations were obtainable in small volumes only and had to be diluted with buffer to provide an adequate volume for treatment purposes. This had the effect of lowering the activity per ml of the preparation. Stage IV pyocine preparations were more easily obtained in large volumes and had a higher activity per ml than the purified pyocine preparations but contained other protein material as well as pyocine. In this experiment, treatment with stage IV and stage VI pyocine preparations was compared.

Five groups of four hairless mice were used and were burned, infected and treated as shown in table 25. The pyocine was applied topically by means of swabs, as described in experiment 2.

The infective dose of Ps. aeruginosa strain P14 was prepared in the following manner: Six NA plates were inoculated with

strain P14 over their entire surface and incubated at 37°C overnight. The growth was scraped off the plates with a bacteriological loop into 10 ml $NB_{20}S$ and centrifuged at 1850 g for 30 min. The supernatant was discarded and the cells were resuspended in 10 ml $NB_{20}S$. This method resulted in an infective dose with a higher viable count (i.e. 3.6×10^9 cells per ml) than that prepared by the standard method (p. 183).

TABLE 25 Schedule of infection and treatment of mice in experiment 3

Group number	Burned	Infected	Treated* with pyocine 1577: Stage VI	Stage IV
1	+	+	-	-
2	+	+	-	+
3	+	+	+	-
4	+	-	-	+
5	+	-	-	-

Key:

+ = regime followed

- = regime not followed

* Treatment was given 4 times at 2 hour intervals on day 2 and 3 times at 2 hour intervals on day 3. Mice that were not treated with pyocine were treated with TIM buffer

The mice were weighed on day 1 before burning and daily thereafter for 8 days. The burns were swabbed before treatment and 1 hour after the last treatment on days 2 and 3 and once on days 4 and 7.

Experiment 4 Treatment of burn infections with stage IV and stage VI preparations of pyocine 5882 and with silver nitrate

Five groups of four hairless mice were burned, infected and treated by the methods described previously, as shown in table 26. Treatment was given 3 times at 2 hour intervals on days 2 and 3.

TABLE 26 Schedule of infection and treatment of mice in experiment 4

Group number	Burned	Infected	Treated with: Stage VI pyocine 5882	Stage IV pyocine 5882	0.5% $AgNO_3$	THM buffer
1	+	+	-	-	-	+
2	+	+	-	+	-	-
3	+	+	+	-	-	-
4	+	+	-	-	+	-
5	+	-	-	-	-	+

Key:

+ = regime followed

- = regime not followed

Impression plates

Small Petri dishes (48 mm diameter) were filled to the brim with nutrient agar. One hour after the last treatment on day 2, two impression plates were taken of the burn on each mouse. One plate was incubated at 37°C overnight to give an indication of the bacterial flora of the burn. The other plate was exposed to chloroform vapour for 5 min. and left open, away from the chloroform,

for a further 10 min. before flooding the surface of the plate with a 4-hour NB culture of strain P14. Excess culture was removed and the lawns were allowed to dry and incubated overnight at 37°C.

Quantitative estimation of Ps. aeruginosa in the burned skin (Based on Lawrence and Lilly, 1972).

On day 8 a surviving mouse in each group was killed by ether and the burned area of skin was excised and shredded into a weighed sterile universal container. The container and skin were weighed together and 10 ml sterile saline per g of tissue were added. The burned skin was homogenised for 10 min. with the container surrounded by ice and water. Tenfold dilutions of the homogenate were made in saline and 0.1 ml of each dilution was spread onto duplicate A9 agar plates. The plates were incubated overnight at 37°C. The viable count of Ps. aeruginosa in the burned skin was estimated from colony counts.

Experiment 5 Treatment of burn infections with a stage VI preparation of pyocine 5882

Three groups of ten hairless mice were burned and two of the groups were infected with Ps. aeruginosa strain P14 by the methods described previously. The uninfected group received sterile $NB_{20}S$. One group of infected mice and the uninfected group were treated with a stage VI preparation of pyocine 5882, 4 times daily, at 2 hour intervals, on the 2nd and 3rd days of

the experiment. The remaining infected group of animals were treated at the same times with sterile THM buffer.

The mice were weighed on day 1 immediately before burning, and daily thereafter for 11 days. The bacterial flora of the burns was monitored by swabbing before the first and 1 hour after the last treatment on days 2 and 3 and once on days 4 and 7. The swabs were cultured on MacConkey agar and representative colonies of Ps. aeruginosa isolated were pyocine typed and checked for resistance to pyocine 5882 by a cross-streaking technique (see p. 159).

Serological investigations

Pyocine neutralisation tests were performed on the sera obtained from mice in the above experiments. The sera were stored at 0°C until they were required, when they were allowed to thaw. Stage II preparations of pyocines 1577 and 5882 were diluted to give activities of 200 units per ml (four times that required to cause confluent lysis of the indicator strain P14). The sera were tested for their ability to neutralise the activity of the pyocine that had been used in the treatment of the mice from which the sera came. Cross-neutralisation tests were not performed.

In each test, doubling dilutions of the serum were prepared in 0.2 ml volumes of saline in a WHO plate. To each dilution was added 0.2 ml of the appropriate pyocine. Serum and pyocine controls were also set up, in which the pyocine and serum respectively were replaced by saline. The contents of the wells

were mixed by carefully agitating the WHO plate and were incubated at 37°C for 1 hour. The serum dilutions were then assayed for pyocine activity by spotting on to lawns of strain P14 on A9 agar plates. The plates were incubated at 37°C overnight and the highest dilution of serum that was capable of neutralising the pyocine activity was recorded. Positive control sera were also included in the tests. These were obtained from rabbits that had been injected with pyocine 1577 or 5882 with Freund's complete adjuvant and bled after five weeks.

RESULTS

Activity of pyocines in vivo

Normal mouse serum did not show any inhibitory activity against Ps. aeruginosa strain P14, but sera from mice that had been injected with pyocine inhibited the growth of strain P14. The results are given in table 27. Control mice were injected with sterile SGB intravenously, intraperitoneally or subcutaneously and bled after 0.5 or 3 hours. On no occasion did the sera from these mice show any inhibitory effect on Ps. aeruginosa strain P14.

TABLE 27 The inhibition of Ps. aeruginosa strain P14 by mouse serum collected at given times after injection of mice with pyocine 1577, 5882 or H108 by various routes

Route of injection	Time at which mouse killed after injection (hours)	Pyocine activity of serum following injection of mice with pyocine: (units per ml)		
		1577	5882	H108
IV	0.5	50,000	25,000	100 - 200
IV	1	25,000-50,000	25,000	100 - 200
IV	3	25,000	12,800-25,000	100
IV	5	12,800-25,000	3200	50
IV	24	800	100-200	0
IP	0.5	50*	3200-6400	0
IP	1	12,800-25,000	6400-12,800	0
IP	3	25,000	12,800-25,000	50
IP	5	12,800	12,800	100
IP	24	1600	800	0
SC	0.5	0	0	0
SC	1	0	0	0
SC	3	50	50	0
SC	5	50	100	0
SC	24	0	200	0

Key:

IV = intravenous
IP = intraperitoneal
SC = subcutaneous
50* = inadequate serum for dilutions

The effect of pyocines on Ps. aeruginosa strain P14 in vivo

(1) By the intraperitoneal route

(A) Using pyocine 1577

From the viable counts of the P14 suspension the infective dose was calculated as 1.4×10^8 organisms. The activity of pyocine 1577 was 1.6×10^6 units per ml.

The weights and deaths of the mice are recorded in table 28. The infective dose of P14 caused 100% mortality of the controls (group 4). The pyocine preparation had no deleterious effects neither did the suspending agent for the cells ($NB_{20}S$). These results also confirm that the trauma of two injections alone was harmless. The mice in groups 2 and 3, which received the bacteria before the pyocine, all died. Mice that received pyocine prior to, or together with, bacteria had a considerably lower mortality.

Examination of the changes in average weights of the surviving groups of mice showed that groups 1, 5 and 6 lost weight initially but regained this over the course of the experiment, whereas groups 7 and 8 showed no weight loss.

Ps. aeruginosa of pyocine type 16 was found in spleen homogenates of all the mice that died during the course of the experiment. No organisms were isolated from the spleens of the animals that survived to the end of the experiment.

(B) Using pyocine 5882

The infective doses of Ps. aeruginosa strain P14 and P14/R

TABLE 28 The numbers of surviving mice and the average weights of the groups of mice on each day of experiment (A)

GROUP		DAY NUMBER:- 1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	Number of mice	6	6	5	5	5	5	5	5	5	5	5	5	5	5
	Av. wt. (g)	19.3	17.1	17.8	19.1	19.8	19.7	19.7	19.9	20.2	20.0	20.6	20.1	20.8	20.9
2		6	0	0	0	0	0	0	0	0	0	0	0	0	0
		19.7	-	-	-	-	-	-	-	-	-	-	-	-	-
3		6	0	0	0	0	0	0	0	0	0	0	0	0	0
		18.7	-	-	-	-	-	-	-	-	-	-	-	-	-
4		6	0	0	0	0	0	0	0	0	0	0	0	0	0
		19.9	-	-	-	-	-	-	-	-	-	-	-	-	-
5		6	6	5	5	5	5	5	5	5	5	5	5	5	5
		19.6	17.7	18.4	20.1	20.7	20.4	20.2	20.6	21.4	20.9	21.2	20.5	21.4	21.8
6		6	5	4	4	4	4	4	4	4	4	4	4	4	4
		20.2	17.9	18.8	20.6	20.9	20.6	20.5	20.5	21.3	20.8	21.3	20.9	21.4	21.6
7		6	6	6	6	6	6	6	6	6	6	6	6	6	6
		19.4	19.6	19.9	20.4	20.2	20.2	20.6	20.5	20.7	19.9	20.8	20.8	21.4	21.5
8		6	6	6	6	6	6	6	6	6	6	6	6	6	6
		19.0	19.7	19.4	20.0	20.0	20.0	20.4	20.3	20.2	20.1	20.6	20.0	20.5	21.2

were calculated from the viable counts of the cell suspensions and were found to be 6.0×10^7 cells and 5.5×10^7 cells respectively. The activity of pyocine 5882 was 5.0×10^4 units per ml.

The numbers of surviving mice and the daily average weights of the groups of mice are recorded in table 29. In table 30 a comparison is drawn between the results of this experiment and those of experiment (A).

The survival of mice infected with strain P14 and treated with pyocine 5882 followed a very similar pattern to that shown when mice were treated with pyocine 1577 (see table 30). But a different pattern of results emerged when pyocine 5882 was used to treat infections caused by strain P14/R: when the pyocine was injected immediately after the P14/R cells all the mice died, but if pyocine treatment preceeded injection by 3 or 6 hours, 3 out of 6 and 2 out of 6 mice survived respectively. Daily average weights of the survivors in these groups (groups 9 and 10) showed that the animals remained unwell and did not recover their initial weight loss over the course of the experiment.

TABLE 29 The numbers of surviving mice and the average weights of the groups of mice injected with Ps. aeruginosa strain P14 or P14/R intraperitoneally in experiment I(B)

Day number	Mouse group number: 1		2		3		4		5		6		7		8		9		10		11		12		13		14		15	
	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b
1	6	20.9	6	20.1	6	20.8	6	20.9	6	28.8	6	27.7	6	27.6	6	26.9	6	28.1	6	25.4	6	29.4	6	28.6	6	27.2	6	27.4	6	26.5
2	5	19.1	0	-	0	-	6	18.9	5	26.6	0	-	0	-	0	-	5	26.3	5	23.7	0	-	0	-	6	26.9	6	27.9	6	25.3
3	5	20.2	0	-	0	-	6	19.2	4	25.8	0	-	0	-	0	-	3	25.8	3	21.3	0	-	0	-	6	26.9	6	27.7	6	26.7
4	5	21.1	0	-	0	-	6	20.6	4	26.7	0	-	0	-	0	-	3	25.6	3	21.1	0	-	0	-	6	27.4	6	28.1	6	26.8
5	5	21.1	0	-	0	-	6	21.3	4	26.9	0	-	0	-	0	-	3	25.6	2	23.4	0	-	0	-	6	26.9	6	28.2	6	26.7
6	5	20.6	0	-	0	-	6	20.8	4	26.9	0	-	0	-	0	-	3	24.9	2	22.6	0	-	0	-	6	27.1	6	28.3	6	26.9
7	5	21.2	0	-	0	-	6	21.4	4	27.3	0	-	0	-	0	-	3	24.5	2	22.6	0	-	0	-	6	27.5	6	28.6	6	27.1
8	5	21.3	0	-	0	-	6	21.4	4	27.1	0	-	0	-	0	-	3	23.5	2	22.0	0	-	0	-	6	27.3	6	28.3	6	26.6
9	5	21.7	0	-	0	-	6	21.7	4	27.4	0	-	0	-	0	-	3	23.3	2	22.3	0	-	0	-	6	27.8	6	29.1	6	27.5
10	5	21.2	0	-	0	-	6	21.2	4	27.5	0	-	0	-	0	-	3	22.9	2	22.2	0	-	0	-	6	27.8	6	29.0	6	27.6
11	5	21.6	0	-	0	-	6	21.9	4	27.6	0	-	0	-	0	-	3	22.6	2	22.4	0	-	0	-	6	27.6	6	29.3	6	27.9
12	5	21.8	0	-	0	-	6	22.3	4	28.0	0	-	0	-	0	-	3	23.2	2	23.0	0	-	0	-	6	28.2	6	29.5	6	28.1
13	5	22.3	0	-	0	-	6	22.7	4	28.2	0	-	0	-	0	-	3	23.6	2	23.8	0	-	0	-	6	28.4	6	29.8	6	28.5
14	5	21.8	0	-	0	-	6	21.9	4	28.3	0	-	0	-	0	-	3	23.9	2	24.4	0	-	0	-	6	28.6	6	30.2	6	28.5

Key: a = number of surviving mice

b = average weight of group of mice (g)

TABLE 30 A comparison of the results obtained after infecting mice with Ps. aeruginosa strain P14 and treating with pyocine 1577 or pyocine 5882

Time of injection of organisms (hours)	Time of injection of pyocine (hours)	Survivors per group of 6 mice after treatment with pyocine:	
		1577	5882
0	0	5	5
0	3	0	0
0	6	0	0
3	0	5	6
6	0	4	4
0	NI	0	0
NI	0	6	6
0*	0	NT	0
0*	3	NT	0
0*	6	NT	0
3*	0	NT	3
6*	0	NT	2

Key:

NT = not tested

NI = not injected

* = mice injected with Ps. aeruginosa strain P14/R

(c) Using pyocine H108

The infective dose of Ps. aeruginosa strain P14 was calculated as 2.8×10^7 organisms. The activity of pyocine H108 was 6.4×10^3 units per ml.

All the mice that were injected with P14 cells died within 8-24 hours of injection whether or not they had also received pyocine H108. The mice receiving pyocine H108 alone all survived.

(2) Infection of burns with Pseudomonas aeruginosa

Experiment 1 The establishment of burn infections

The four infected mice began to lose weight within 48 hours of the start of the experiment; two mice died on day 5 and one mouse died on day 7. The 4th mouse survived. The average weight of the uninfected group did not change greatly over the course of the experiment.

The viable count of the suspension of Ps. aeruginosa strain P14 was 2.1×10^8 organisms per ml and thus the infective dose per burn was approximately 2.1×10^7 bacteria. Burns that had been inoculated with Ps. aeruginosa strain P14 began to show signs of infection after about 48 hours (fig. 17). The burn became red and inflamed and also had a greenish tinge. Burns on the uninfected mice reddened slightly but were not inflamed and soon began to contract and dry up. The eschars on the uninfected burns began to lift after four days whereas the eschar on the surviving infected mouse did not begin to lift until 15 days after the start of the experiment.

Cultures from swabs of the burns taken on day 3 and day 7 showed that all the burns of the infected mice were colonised by Ps. aeruginosa and two of the mice were also contaminated with Proteus species. None of the uninfected mice had any Ps. aeruginosa on their burns but 3 out of 4 were colonised by staphylococci and 3 out of 4 by Proteus species; such colonisation of the "uninfected" group did not appear to affect the mice and all the burns healed about 15 days after burning.

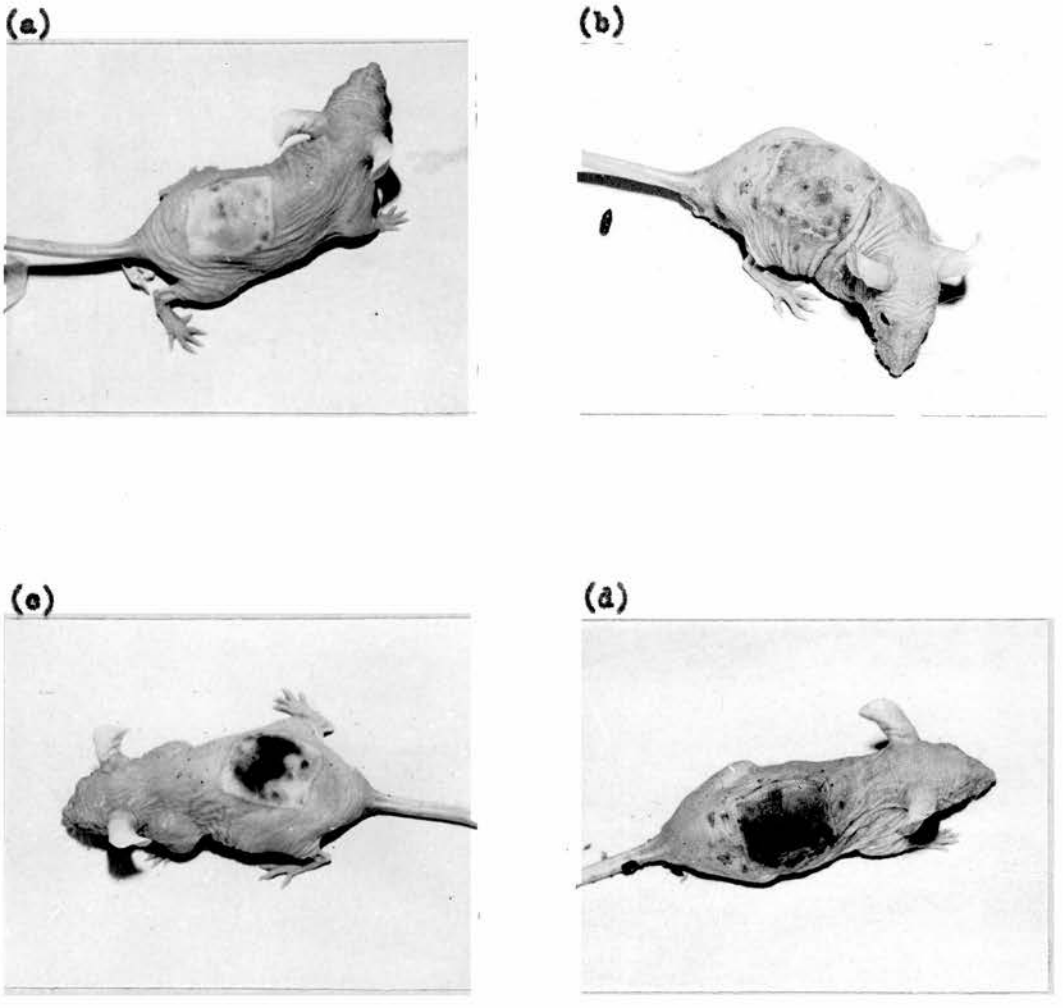


FIGURE 17 The appearance of burned mice infected with *Pseudomonas aeruginosa* strain P14

(a) One day after burning and infecting

(b) Two days after burning and infecting

(c) Four days after burning and infecting

(d) At death, five days after burning and infecting

Experiment 2 Treatment of infected burns with stage VI pyocine 1577

The viable count of the Ps. aeruginosa strain P14 suspension used in this experiment was 9.0×10^7 organisms per ml. Each mouse received approximately 0.1 ml of this suspension, thus the infective dose was 9.0×10^6 organisms per burn.

Stage VI pyocine 1577 suspended in THM buffer was used for treatment. The average activity of the suspension over the three treatment days was 4.2×10^4 units per ml.

The average weights of the groups of mice are given in table 31. The positive controls i.e. mice that were infected but not treated, showed the greatest loss in weight and one mouse died three days after infection. Post-mortem cultures of the spleen homogenate and of swabs taken of the peritoneal and thoracic cavities revealed abundant growth of Ps. aeruginosa (pyocine type 16). Animals in group 1, which were infected and treated topically with pyocine, showed a slight loss in weight initially but recovered later in the experiment.

The bacterial flora of the burns was monitored by daily swabbing. Before infection with Ps. aeruginosa most of the burns produced a scanty growth of Gram-positive cocci. The uninfected burns maintained this flora and one also became colonised by Proteus species. Twenty-four hours after infection with Ps. aeruginosa strain P14, the burns showed some growth of this organism on culture and by 48 hours all the infected burns yielded a heavy growth of Ps. aeruginosa of pyocine type 16.

TABLE 31 Daily average weights of groups of mice that were burned, infected with Ps. aeruginosa strain P14 and treated with pyocine 1577

	Group	Average weight of group (g) on day: 1	2	3	4	5	7	8	9	10	11	12	13	14	No. of eschars off per group
1	Infected Treated topically	30.4	30.5	29.9	29.8	29.8	29.8	30.4	30.8	31.2	30.8	31.3	31.6	31.3	$2\frac{1}{2}$
2	Infected Treated IV	31.5	31.6	31.2	30.4	30.9	31.5	32.0	32.1	32.3	31.8	32.6	32.2	31.7	$3\frac{1}{2}$
3	Infected Not treated	33.8	33.8	33.2	35.5*	35.3*	35.2*	35.9*	35.9*	36.4*	34.9*	35.7*	35.5*	34.9*	2 +
		36.0*	35.9*	35.7*											
4	Not infected Not treated	31.8	32.1	32.7	32.3	32.3	31.8	32.3	32.8	32.7	32.0	32.8	33.0	32.4	$2\frac{1}{2}$
5	Not infected Treated topically	27.7	26.8	26.5	27.0	26.8	26.8	27.2	27.4	27.6	26.7	27.7	27.0	27.2	$\frac{1}{2}$
6	Not infected Treated IV	33.3	33.2	33.5	32.9	33.1	32.8	33.3	33.4	33.9	32.9	33.4	32.9	32.9	$2\frac{1}{2}$

Key:

* Average weight of 3 surviving mice

\+ Group of 3 mice

IV intravenously

Despite topical or intravenous therapy with pyocine 1577, there was no apparent reduction in the amount of Ps. aeruginosa colonisation of the burns. The level of Ps. aeruginosa on the burns began to decrease around the 12th day as healing progressed.

At the end of the experiment the number of burns that had lost their eschars was recorded (see table 31). The results suggested that infection of burns with Ps. aeruginosa did not prevent healing.

Experiment 3 Treatment of burn infections with stage IV and stage VI preparations of pyocine 1577

The infective dose of Ps. aeruginosa strain P14 was prepared from solid culture rather than broth culture because this method resulted in a higher viable count. In this experiment, the viable count of the cell suspension was 3.6×10^9 organisms per ml and thus the approximate infective dose per mouse was 3.6×10^8 organisms.

Two preparations of pyocine 1577 from different stages of the purification procedure were used. The stage IV pyocine had an activity of 3.8×10^5 units per ml and the stage VI preparation had an activity of 1.2×10^4 units per ml.

The growth of Ps. aeruginosa on the infected burns was monitored before and after pyocine treatment on days 2 and 3 of the experiment and also on days 4 and 7. The infected mice were all heavily colonised by Ps. aeruginosa (pyocine type 16) before treatment commenced. Cultures from swabs taken 1 hour

after the last treatment on days 2 and 3 showed that the amount of Ps. aeruginosa on the burns had been reduced in both the stage IV and stage VI pyocine-treated groups. However samples from the burns taken at the beginning of days 3 and 4 showed that the Ps. aeruginosa had returned to its pre-treatment level.

In spite of the high infective dose, the positive control group of mice, which were infected but not treated, had only a 50% mortality rate. Similarly in the group treated with stage VI pyocine 1577, two of the four mice died. Mice that were treated with stage IV pyocine all survived as did the uninfected controls.

Experiment 4 Treatment of burn infections with stage IV and stage VI preparations of pyocine 5882 and with silver nitrate

The infective dose of Ps. aeruginosa strain P14 used in this experiment was approximately 4.1×10^8 organisms per burn. The activities of the stage IV and stage VI preparations of pyocine 5882 were 1.6×10^5 units per ml and 1.2×10^4 units per ml respectively.

The mortality of the burned mice is given in table 32. The mice in group 3, which were treated with stage VI pyocine 5882, showed the best survival, but their burns remained colonised by Ps. aeruginosa.

Impressions of the burns were made on agar to examine the bacterial flora after treatment and to try to demonstrate the

TABLE 32 Mortality of burned mice after infection with Ps. aeruginosa strain P14 and treatment with pyocine 5882 or silver nitrate solution

Group number	Infected with Ps. aeruginosa P14	Treatment	Number of mice		Mean time to death (days) of mice that died
			Died	Survived	
1	+	THM buffer	3	1	3.0
2	+	Stage IV pyocine 5882	3	1	3.0
3	+	Stage VI pyocine 5882	1	3	3.0
4	+	0.5% silver nitrate soln.	3	1	3.3
5	-	THM buffer	0	4	. . .

Key:

+ = burns infected

- = burns not infected

presence of active pyocine on the burn surface. The latter was shown by the inhibition of growth of strain P14 on a sterilised impression plate. The results are shown in table 33.

TABLE 33 The presence of Ps. aeruginosa and of inhibitory activity on mouse burns treated by various methods

Group number	Burn infected with Ps. aeruginosa	Treatment	1 hour after final treatment on day 2				
			Growth of Ps. aeruginosa from each burn				Inhibition of lawn of P14
			Mouse: 1	2	3	4	
1	Yes	THM buffer	+	+	+	+	NI
2	Yes	Stage IV pyocine 5882	±	-	-	-	I
3	Yes	Stage VI pyocine 5882	±	+	±	±	NI
4	Yes	0.5% $AgNO_3$	+	+	+	+	NI
5	No	THM buffer	-	-	-	-	NI

Key:

+ = growth of Ps. aeruginosa

± = scanty growth of Ps. aeruginosa

- = no growth of Ps. aeruginosa

NI = lawn of strain P14 not inhibited

I = inhibition of lawn of P14

On day 8, the burned skin was excised from one mouse in each group. Viable counts of Ps. aeruginosa were made from homogenates of the burns and the results are given in table 34.

TABLE 34 Viable counts of Ps. aeruginosa in homogenates of burned skin

Mouse from group number	Wt. of burned skin (g)	Viable count of Ps. aeruginosa per g of burned skin
1	0.33	4.5×10^8
2	0.54	6.6×10^8
3	0.53	4.0×10^8
4	0.60	2.8×10^8
5	0.41	0

Experiment 5 Treatment of burn infections with a stage VI preparation of pyocine 5882

The infective dose of Ps. aeruginosa strain P14 was prepared from solid culture rather than broth, and the average viable count per mouse was 2.2×10^8 organisms. The activity of the stage VI preparation of pyocine 5882 used for treatment was 1,600,000 and 400,000 units per ml on days 2 and 3 respectively.

The numbers of surviving mice in each group are shown in table 35. No Ps. aeruginosa was isolated from the uninfected mice and these remained healthy throughout the experiment. Cultures of swabs taken before and after pyocine treatment of

infected burns did not reveal any obvious reduction in the amount of Ps. aeruginosa isolated from the burns after pyocine treatment. The representative colonies of Ps. aeruginosa that were picked, were found to belong to pyocine type 16 and had retained their sensitivity to pyocine 5882, as indicated by cross-streaking.

At the end of the experiment, 60% of the mice whose burns were infected with Ps. aeruginosa strain P14 and treated with pyocine 5882 were dead; by comparison, 70% of the infected but untreated mice had died. The mean time to death of the treated mice was 6.3 days compared with 7.3 days for the untreated group.

TABLE 35 Survival of burned mice infected with Ps. aeruginosa strain P14 and treated with a stage VI preparation of pyocine 5882

Condition of mice	Number of animals (per group of 10) alive on day: 1	2	3	4	5	6	7	8	9	10	11	Mean time to death (days)
Infected & treated	10	10	8	6	6	5	5	5	5	5	4	6.3
Infected & untreated	10	10	8	8	8	8	8	8	5	4	3	7.3
Uninfected & untreated	10	10	10	10	10	10	10	10	10	10	10	. . .

Serological investigations

Neutralisation tests were carried out to demonstrate the presence of specific antibodies in the sera of mice that had been treated with pyocine. The highest dilution of serum that was capable of neutralising the pyocine activity was recorded and the results are given in tables 36 and 37. (N.B. 1 in 128 was the highest serum dilution tested).

TABLE 36 Neutralisation titres of sera from groups of mice injected intraperitoneally with Ps. aeruginosa strain P14 and pyocine 1577 or 5882

Mouse group number	Time of injection on day 1: 0	3	6 hours	Neutralisation titre of sera from group
1	P14 1577*	-	-	1/128
5	1577	P14	-	1/128
6	1577	-	P14	1/128
7	1577	THM buffer	-	1/32
8	$NB_{20}S$	$NB_{20}S$	-	0
1	P14 5882*	-	-	1/128
4	5882	P14	-	1/128
5	5882	-	P14	1/128
9	5882	P14/R	-	1/128
10	5882	-	P14/R	1/128
13	5882	THM buffer	-	1/64
14	$NB_{20}S$	$NB_{20}S$	-	0

Key:

P14 = Ps. aeruginosa strain P14; cells suspended in $NB_{20}S$

P14/R = Ps. aeruginosa strain P14/R (pyocine 5882 - resistant) suspended in $NB_{20}S$

1577 = Pyocine 1577 suspended in THM buffer

5882 = Pyocine 5882 " " " "

$NB_{20}S$ = 20% nutrient broth in saline

* mice in these groups were all injected with strain P14 into one side of the peritoneal cavity and then with pyocine 1577 or 5882 into the other side

TABLE 37 Neutralisation titres of sera from groups of mice that were burned, infected with Ps. aeruginosa strain P14 and treated with pyocine 1577

Mouse group number	Regime followed	Neutralisation titre of sera of group
1	Burn. Infect. Topical pyocine treatment	0
2	Burn. Infect. Systemic pyocine treatment	1/128
3	Burn. Infect. No treatment	0
4	Burn. Infect. No infection of treatment	0
5	Burn. No infection. Topical pyocine treatment	0
6	Burn. No infection. Systemic pyocine treatment	1/128

DISCUSSION

If pyocines are to be useful as therapeutic agents, they must retain their inhibitory activity *in vivo* in the presence of serum and tissue exudate. Higerd, Baechler and Berk (1967) found that aged normal human or rabbit sera enhanced the activity of pyocine C9 fourfold *in vitro*. The inhibitory activity of pyocines 1577, 5882 and H108 in mouse serum was examined. Since the sera from control mice did not inhibit the growth of *Ps. aeruginosa* strain P14, the inhibitory activity of the test sera was assumed to be due to the injected pyocine.

The high molecular weight pyocines 1577 and 5882 gave a similar pattern of results: following intravenous administration of pyocine, the inhibitory activity of the serum was greatest in the first sample (i.e. after 0.5 hour) and gradually decreased over the course of the experiment (24 hours). Intraperitoneal injection of pyocine resulted in a rise in the serum inhibitory titre to a maximum at 3 hours after injection, followed by a decrease during the next 21 hours. Little inhibitory activity could be detected in the sera of mice that had received pyocine subcutaneously.

Due to difficulties in preparation, pyocine H108 was administered at a much lower titre than pyocines 1577 and 5882 and very little inhibitory activity could be detected in the sera of the test animals.

Thus it was concluded that pyocine preparations retain at least some of their inhibitory activity in the presence of the body fluids of the test animal.

The effects of pyocines in the presence of Ps. aeruginosa in vivo were examined in two experimental systems; by intraperitoneal administration of bacteria and pyocine, and by topical application of pyocine to infected burns. Intraperitoneal injection of a suitable dose of Ps. aeruginosa strain P14 results in rapid death, but mice treated with pyocine 1577 before they were injected with strain P14 showed a markedly improved survival rate compared with animals that did not receive any pyocine or that were treated after they were infected. It has been shown that pyocine remains active in the blood for some hours after intraperitoneal administration and where it was present in mice when the infective dose was given, the pyocine was apparently capable of reducing the number of bacteria to a level that could be dealt with by the animals' defence mechanisms, and most of the mice survived. Similarly, mice that received bacteria and pyocine simultaneously showed good survival.

However, the pyocine was unable to prevent the fatal outcome of infection when it was given three or six hours after the infective dose. The P14 culture was centrifuged and resuspended in fresh broth before injection to reduce the carry-over of any toxic growth products. Thus the results suggest

that the lethal products of the bacteria were produced soon after inoculation and a reduction in bacterial numbers at a later stage did not prevent death.

Treatment of intraperitoneal infections with the filamentous pyocine 5882 gave very similar results. Mice that were treated with pyocine before they were infected with strain P14 generally survived but mice that were infected first and treated with pyocine later all died. When the pyocine and bacteria were given at the same time, five of the six mice survived. Pyocine 1577 used in the experiment had an activity of 1.6×10^6 units per ml whereas pyocine 5882 had an activity of only 5.0×10^4 units per ml, but this difference was not reflected in the results of the comparative experiments.

Ps. aeruginosa strain P14/R was isolated from a colony of strain P14 that had grown in the presence of pyocine 5882 *in vitro*. However, it appeared that some of the cells in the infective dose of P14/R were sensitive to the pyocine *in vivo*, thus the viability of the infective dose was reduced and some of the mice survived. When pyocine 5882 and P14/R were injected simultaneously all the mice died, compared with only one death in the group injected with the pyocine and P14 cells simultaneously. This suggests that the inhibition of P14/R cells by pyocine 5882 may be slower than that of P14 cells.

Compared with the protective effect of pyocines 1577 and 5882 on mice infected with strain P14, the results with pyocine H108 were unfavourable, probably because of the low inhibitory activity of the pyocine suspension. The infective dose of

Ps. aeruginosa strain P14 was lethal to all the mice, both in the presence and absence of pyocine H108.

Infections initiated by intraperitoneal inoculation of Ps. aeruginosa are easy to establish but are poor representatives of natural pseudomonas infection, whereas the infection of experimentally-induced burns closely mimics the human counterpart.

The method of burning mice was based on that described by Jones, Jackson and Lowbury (1966) and was readily performed. The only problem was in ensuring that the whole surface of the brass block was in contact with the mouse's skin. The use of hairless mice had the advantage that it made depilation unnecessary. The mice did not appear to differ from normal mice except in their lack of hair.

Jones and his colleagues (1966) reported a mortality rate of 71% following Ps. aeruginosa infection of mouse burns. Experiment 1 in this series gave a similar result although the number of animals involved was small. Burning, without subsequent infection, produced no deaths in any of these experiments. The burns that were not infected with Ps. aeruginosa frequently became contaminated with Gram-positive cocci, but these did not appear to have any adverse effects on the mice. Occasionally Proteus species were also cultured from the burns. This was probably the result of contamination of the burn from the faeces of the mouse. Sampling of the faeces before the start of an experiment revealed a number of lactose non-fermenting Gram-negative bacilli, including Proteus.

However, Proteus colonisation of the burns did not seem to have any deleterious effects on the mice.

When Ps. aeruginosa infects a burn, the bacteria gradually invade the deeper tissues and death results from septicaemia. In experiment 2, two routes of pyocine treatment of infected burns were examined. Topical pyocine treatment was aimed at reducing the surface infection with Ps. aeruginosa, while intravenous therapy was designed to counteract septicaemic spread of the bacteria. However, the results showed that the infective dose of Ps. aeruginosa was too small and the mortality of the infected but untreated mice was very low. In view of the lack of clear positive controls, it was difficult to draw any conclusions on the efficacy of pyocine treatment. The topically-applied pyocine may have reduced the numbers of Ps. aeruginosa on the burn transiently, but this reduction was not obvious from the daily cultures. In later experiments, the frequency of sampling was increased to give a better picture of changes in the numbers of bacteria on the burn. Uninfected controls that were treated with pyocine did less well than uninfected mice that were not treated, as judged by the daily average weights of the groups. This suggested that the pyocine treatment alone may have had some adverse effects. Although the infected mice did not die, their burns remained colonised by Ps. aeruginosa for some time, but this did not apparently prevent normal healing of the burn wound.

The technical problems of pyocine therapy centred around the production of adequate volumes of purified pyocine of high

activity. A fresh batch of pyocine was prepared for each experiment and the preparation time was about ten days (see Section 2). After the final purification stage by DEAE-cellulose ion-exchange chromatography, the pyocine was dispersed in a large volume (c. 80 ml) of buffer and had a low activity per ml. Ultracentrifugation was used to concentrate the dilute pyocine suspension and as a result, only small volumes of purified pyocine were obtained. Dilution of the concentrate was necessary to provide enough pyocine for the experiment and consequently the activity per ml of the purified (stage VI) pyocine suspension used for treatment was rather low (c. 1.2×10^4 units per ml). Pyocine suspensions of higher activity (c. 2.0×10^5 units per ml) were obtained in larger volumes at the intermediate step in the purification procedure (stage IV). These preparations had not been subjected to column chromatography and thus were contaminated with other protein material. The therapeutic potential of such pyocine preparations of lower purity but higher unit activity was compared with that of the purified pyocine, in experiments 3 and 4. The requirement for pyocine was reduced by examining topical treatment only.

The method of applying pyocine to the burn surface i.e. with a cotton-wool swab soaked in pyocine, was far from quantitative. It was confirmed that the pyocine activity was not destroyed on the swab, but the volume of pyocine transferred onto the burn was uncertain. The swab may also have been

responsible for removing organisms from the surface of the burn. There was no way of preventing this, but the error was common to all the mice. A sterile swab was used for each treatment of each mouse to prevent contamination of the stock pyocine suspension.

The small study in experiment 1 showed that an infective dose of 2.1×10^7 Ps. aeruginosa strain P14 was lethal, but in further work the doses were found to be too small to cause death of the positive controls. In order to show that pyocine treatment could prevent death of infected mice, it was necessary that the untreated, infected mice (i.e. the positive controls) should die. To try to achieve this end, the viable count of the infective dose of Ps. aeruginosa was raised approximately twentyfold in experiments 3 and 4.

Experiment 3 showed that the topical treatment of burn infections with pyocine 1577 did reduce the amount of Ps. aeruginosa on the burn surface. But this reduction was transient and within 16 hours, the Ps. aeruginosa had returned to its pre-treatment level. Although the infective dose had been increased, there was only a 50% mortality among the positive controls. Two of the four infected mice, treated with stage VI pyocine 1577, died in spite of the temporary reduction of Ps. aeruginosa on the burn by the pyocine.

Pyocine 5882 was used for treatment in experiment 4, and was compared with silver nitrate solution which is recommended for the treatment of human burns (Cason and Lowbury, 1968).

Superficially the results of this experiment were favourable to pyocine therapy. There was a high mortality rate in all the infected groups of mice except those treated with stage VI pyocine 5882, in spite of the low activity of the pyocine preparation (1.2×10^4 units per ml). Impression plates were used to give a direct image of the surface of the burn after pyocine treatment. The results showed that there was a significant reduction in Ps. aeruginosa on the burn surfaces one hour after treatment with stage IV pyocine 5882 and a slight reduction after treatment with the stage VI pyocine. Treatment with 0.5% silver nitrate solution did not reduce the level of Ps. aeruginosa on the burn. A second impression of each burn was taken, sterilised and lawned with Ps. aeruginosa strain P14, to see whether there was any demonstrable inhibitory activity remaining on the burn one hour after pyocine treatment. The results suggested that there was some pyocine activity present on the burns treated with stage IV pyocine. No inhibitory activity was demonstrable on any of the other burns.

The results from the impression plates support the idea that the survival of the infected mice treated with stage VI pyocine 5882 was fortuitous and may have been due to a lower level of infection. The numbers of Ps. aeruginosa in the burns of the surviving mice in each group were estimated by excising the burned skin, homogenising it and preparing a viable count. The samples were all taken from mice that had

survived until the 8th day of the experiment and the results showed that these burns were all infected with approximately the same number of Ps. aeruginosa. This suggested that this level of infection in the burn (c. $2.0 - 7.0 \times 10^8$ organisms per g skin) was not lethal. Quantitative estimation gives a much more accurate picture of Ps. aeruginosa in the burn and it would be desirable to follow the changes in numbers of bacteria during pyocine treatment by this method. It would however, involve large numbers of mice and thus large volumes of pyocine, to obtain meaningful results.

Experiment 5 was designed to clarify the results of the previous experiment on the efficacy of topical pyocine 5882 treatment on burns infected with Ps. aeruginosa strain P14. The numbers of mice in each group were increased in an effort to overcome the variability in the "take-rate" of the infections. The full preparative procedure for pyocine 5882 was carried through twice to provide an adequate volume of stage VI pyocine, and treatment was commenced 24 hours after infection. Only eight doses of pyocine per mouse were available and these were spread over 2 days. However, from about 48 hours after infection, Ps. aeruginosa starts to invade deeper into the burned tissue and thus becomes inaccessible to topical treatment. The results of the experiment show fairly conclusively that topical treatment with pyocine 5882 did not significantly improve the chances of survival of burned mice infected with Ps. aeruginosa strain P14.

One of the problems associated with the therapeutic use of pyocines is their antigenicity. Pyocines have been shown to be good antigens when injected with adjuvants into animals, but it is not known to what degree they are antigenic when administered alone. In the course of these experiments on the therapeutic uses of pyocines in Ps. aeruginosa infections, serum was collected from some groups of mice and tested subsequently for the presence of antibodies to the pyocine with which the animals had been treated.

In experiments where the infective dose of Ps. aeruginosa strain P14 and the pyocine were given intraperitoneally, it was found that in mice that survived, a single injection of pyocine caused the development of neutralising antibody within 13 days. Control mice that received pyocine only and were not infected, showed a somewhat lower antibody response than mice that had also received organisms. It may be that the Ps. aeruginosa acts in the manner of an adjuvant and increases the antibody response to the pyocine. Unfortunately, the neutralising capacity of dilutions of serum greater than 1 in 128 was not examined and the actual neutralisation titre of the sera may have been much higher. The sera were not tested for antibodies to the infecting strain P14.

The treatment of Ps. aeruginosa burn infections with pyocine 1577 resulted in little antibody development, and it was only in those mice that had received pyocine 1577 intravenously that pyocine-neutralising antibodies were detected. Topical pyocine treatment did not elicit an antibody response.

GENERAL DISCUSSION

GENERAL DISCUSSION

Pseudomonas aeruginosa is often regarded as a saprophyte, but in the hospital environment it frequently assumes the role of a dangerous pathogen which takes a particular delight in attacking debilitated patients. Since the introduction of broad-spectrum antibiotics, a disturbing increase in the incidence of Ps. aeruginosa infections has been noted (Finland, Jones and Barnes, 1959; Asay and Koch, 1960), and has brought to light the problems associated with the eradication of this organism. Once established, Ps. aeruginosa infections are difficult to cure because of the organism's natural resistance to many antimicrobial agents and its ability to develop or acquire resistance to the few effective agents that are currently available, e.g. the antibiotics gentamicin and carbenicillin. Even in surface infections, such as those of burns, where more drastic antimicrobial therapy can be used, Ps. aeruginosa infection remains a serious hazard. Since the organism appears to be undaunted by our therapeutic efforts, the consideration of an alternative type of antibiotic treatment seemed reasonable. The idea of pyocine therapy has already been broached and the published reports are favourable (Bird and Grieble, 1969; Merrikin and Terry, 1972). Thus it seemed feasible to extend these preliminary studies and examine the therapeutic applications of pyocines in greater depth.

Ps. aeruginosa is known to be capable of elaborating three kinds of pyocines which may be differentiated by their molecular

weight and morphology. Neither Bird and Grieble (1969) nor Merrikin and Terry (1972) defined the kind of pyocine they were dealing with and it is difficult to make a critical assessment of their results. In this investigation, a representative of each kind of pyocine was selected; contractile, filamentous and small, each produced by a different pyocinogenic strain, but all inhibitory to Ps. aeruginosa strain P14. This indicator strain was chosen because it was known to be virulent to mice when applied to experimentally-induced burns (Jones, Jackson and Lowbury, 1966). A considerable proportion of the study involved the characterisation of the selected pyocines and an examination of suitable purification and concentration techniques. Before therapeutic investigations were undertaken, in-vitro experiments were carried out to examine the interactions between the selected pyocines and indicator strain P14. Some potential problems associated with the therapeutic use of pyocines were brought to light; it was difficult to eradicate strain P14 completely by pyocine treatment and the emergence of pyocine-resistant variants was noted.

An ideal therapeutic agent may be considered as requiring the following properties:

(a) It should specifically attack the pathogen while being non-toxic to the host.

(b) It should be stable and active in the presence of serum and tissue exudate.

(c) It should not encourage the development of resistance.

(d) It should preferably be cheap and easy to use.

In the light of the results of this investigation, a comparison may be drawn between the pyocines and an ideal therapeutic agent.

Pyocines are by definition extremely specific in their inhibitory activity and only attack strains within the genus that possess suitable receptors. This study was confined to a single indicator strain and the spectra of activity of the selected pyocines were not examined. However, it would appear that a single pyocine preparation would have a very limited range of activity and the sensitivity of an infecting strain to a number of pyocines would have to be tested before treatment could commence.

The toxicity of pyocines to animals has not been satisfactorily established. Homma and Suzuki (1964) found that a cell wall-associated protein with pyocine activity had some toxic properties. Bird and Grieble (1969) reported in their therapeutic studies that the administration of pyocine alone to chick embryos resulted in an 11% mortality. The nature of the pyocine that Bird and Grieble used is not clear from their results (1969) but other workers (Higerd, Baechler and Berk, 1967; Govan, 1968) who have studied the contractile pyocines have found them to be completely non-toxic. Unfortunately, the results presented here are not conclusive and certain preparations of the contractile pyocine 5893 were toxic to mice. Therefore this pyocine was replaced by pyocine 1577 which was also of the contractile type, but in a purified form had no adverse effects on mice. The filamentous pyocine 5882 did not

exhibit any toxic properties, but injection of preparations of the small pyocine H108 into mice did result in some deaths. The problems of toxicity associated with pyocines may be due to impurities, such as enzymes, which are present in the preparations and with further studies of purification procedures it may be possible to produce non-toxic products.

However, the problems of antigenicity are not so easily conquered. A single therapeutic dose of pyocine given intraperitoneally was sufficient to elicit an antibody response in mice. This would severely limit systemic pyocine therapy, and since there are serological relationships between different pyocines within the same class (Homma, Goto and Shionoya, 1967) treatment with an alternative pyocine would not necessarily solve the problem. The outlook for topical application of pyocine is somewhat brighter in this respect since a short course of treatment did not result in antibody production.

The retention of pyocine activity in the presence of serum was examined by injecting mice with pyocine by various routes and examining the serum for inhibitory activity. It was concluded that the pyocines retained some of their activity for at least 24 hours. The serum inhibitory titre following the intravenous administration of pyocine fell progressively over this time, whereas pyocine given intraperitoneally reached its maximum serum level about three hours after injection and then decreased. The time-lag presumably reflects the time involved in clearing the pyocine from the peritoneal cavity into the bloodstream.

The way in which this is effected is not understood, but it is apparently more efficient than the delivery of subcutaneous pyocine to the blood judging from the low levels of pyocine activity in the serum following subcutaneous injection.

The small pyocines are sensitive to the action of proteolytic enzymes and it might be suggested that they would be inactivated by such enzymes in the body. However, Braude and Siemienski (1965) found that colicine activity, which is also sensitive to proteolytic enzymes, was not destroyed in the serum of mice. From the levels of inhibitory activity present in mouse serum after intravenous administration of the small pyocine H108, it would appear that this pyocine was also unaffected by the proteolytic enzymes in vivo.

The resistance of Ps. aeruginosa to many antimicrobial agents is the prime cause for the triumph of this organism in the hospital environment. Although Patterson (1965) found that pyocine-resistant mutants were rare, their existence has been described by several other authors. Kageyama, Ikeda and Egami (1964) suggested that in a sensitive culture, treated with pyocine, there was a high frequency of mutation to pyocine resistance. Govan (1968) found that cells that were apparently resistant to pyocine attack reverted on subsequent re-testing and stable mutants were difficult to obtain. Tolerant mutants i.e. cells which are able to absorb small pyocines but are insensitive to their action, have been reported by Holloway (1971).

In this investigation, it was found that pyocine-resistant

variants emerged rapidly in a culture of strain P14 exposed to pyocine *in vitro*, and these appeared to be stable on subsequent testing. However, cultures of strain P14 obtained from infected animals that had been treated with pyocines did not appear to have developed resistance during the course of treatment. The study of pyocine resistance *in vivo* was superficial and the rapid emergence of resistant mutants *in vitro* is foreboding.

The preparation of purified pyocine was a rather lengthy procedure and the poor "shelf-life" of the purified pyocines necessitated preparing a fresh batch for each course of treatment. To date, suitable methods for storing pyocines in their active state have not been clearly defined, but further research may solve this problem.

From this review, pyocines emerge with rather dubious possibilities as therapeutic agents but some of the problems described may be surmountable by further research. However, from the results of the therapeutic studies reported here, the actual value of pyocine therapy is doubtful. Systemic administration of pyocine could only prevent the fatal outcome of infection if it was given before or simultaneously with the infective dose, and this is a somewhat impractical form of treatment. Initially, topical therapy seemed to be a more likely outlet for pyocines, but it was found that the inhibition of *Ps. aeruginosa* by the pyocines tested was insufficient to prevent invasion of bacteria from the infected burn and subsequent septicaemia and death.

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